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THE ROLE OF NEWLY DISCOVERED EXOTOXIN (S TOXIN) IN PSEUDOMONAS AERUGINOSA INFECTIONS

Annual Report

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SUMMARY

A. Our long term goal is to determine the role to exotoxin S in humans infected with Pseudomonas aeruginosa and develop vaccines to reduce the morbidity and mortality associated with these infections. During the periods of the project covered in this annual report (August 1, 1978-July 31, 1979) we have:

(1) Completed our initial studies of S production in vitro and in vivo. One publication and a draft of a second manuscript in preparation are appended.

(2) Initiated a study on the incidence of toxin S production by clinical isolates of P. aeruginosa. The most intriguing observation has been that bacteremic patients infected with S producing strains have a much higher mortality rate (71%) compared to those infected with strains of P. aeruginosa that don't produce S (43% mortality rate).

(3) We have tested the virulence of strain Ps 388 and a protease deficient mutant Ps 388-6 in four different animal models. Both Ps 388 and Ps 388-6 produce S but not toxin A. Both strains were virulent in the burned mouse model and a rabbit model which mimics localized skin infections. However both strains were avirulent in a mouse eye model and a neutropenic rabbit model.

(4) We have studied the assay condition used for measuring the enzymatic activity of S. Parameters investigated were pH, ionic strength, buffer composition, metal requirements and the effects of detergents. The optimum conditions and ionic requirements were found to be different for S than those reported for A.

(5) We have continued our studies on the purification and characteristics of S. We have investigated two purification schemes based on conventional protein chemistry methods. We have further characterized S with respect to interaction with antisera, molecular weights and substrate specificity.

(6) We have purified several batches of toxin A and diphtheria toxin. Approximately 80% of these purified toxin preparations have been sent to Drs. J. Sadoff and C. Alving (Walter Reed Army Inst. of Research) and Dr. W. Brodsky (Mt. Sinai Med. Center) for collaborative studies supported by the army. One paper (#3 below) and one abstract (#4 below) have resulted from these collaborations this year.

B. Publications resulting from this research (copied appended).

1. Bjorn, M.J., Pavlovskis, O.R., Thompson, M.R., and Iglewski, B.H. Production of Exoenzyme S during Pseudomonas aeruginosa infections of burned mice. Infect. Immun. 24:837,1979.

2. Bjorn, M.J. and Iglewski, B.H. Factors that influence the production of Pseudomonas aeruginosa Exoenzyme S. To be submitted.

3. Brodsky, W.A., Sadoff, J.C., Durham, J.H., Ebrenspeck, G., Schachner, M. and Iglewski, B.H. Effects of pseudomonos toxin A, Diphtheria toxin and cholera toxin on the electrical parameters of the turtle bladder. Proc. Nat. Acad. of Sci. U.S.A. 76:3562, 1979.

4. Alving, C.R., Iglewski, B.H., Urban, K.A., Moss, J. and Sadoff, J.C. Binding of diphtheria toxin to phospholipids in liposomes. F.A.S.E.B. Abstracts, 1979.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

During the course of this work the authors were greatly assisted by Dr. Michael Bjorn, Mr. Dennis Ohman, Mr. Jack Lile, Mrs. Pamela Sokol and Mrs. Joan Rittenberg. Their help is deeply appreciated. Portions of this research were done in collaboration with Drs. J. D. Sadoff, Alan Cross, and Carl Alving, WRAIR, Washington, D.C., O.R. Pavlovskis, ONR, Washington, D.C., and Dr. E. Ziegler, U. of California at San Diego.

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I. Production of toxin S and a comparison of the virulence of S producing strains in various animal models.

A. Introduction

Our initial studies on the production of S *in vitro* and *in vivo* have been continued. We have compared the effects of various chelators added to the growth medium on S production. We extended our characterization of S produced *in vivo*, in burned mice infected with strain Ps 388. We have begun a study on the incidence of S production by *P. aeruginosa* strains isolated from patients with bacteremia. Finally, our observations that strain Ps 388 was virulence in the burned mouse model were extended by examining the virulence of this strain in three additional animal models.

B. Materials and Methods

1. Previously described methods. Most of the methods utilized in these studies were described in detail in last years progress report. These include standard culture media, deferration and iron determinations, bacterial strain characterization and maintenance, ADP-ribosyl transferase assay, protease determinations and the burned mouse model.

2. Determination of the toxin phenotype of clinical isolates. Strains isolated from patients with *P. aeruginosa* bacteremia were provided by Drs. J. Sadoff and A. Cross (WRAIR). These isolates were grown in the trypticase soy broth dialysate (TSB_D) with and without NTA (final concentration 10mM at 32°C) for 22 hr. (1) culture supernatants were tested in the ADP-ribosyl transferase assay (2) with and without prior incubation with 4M urea and 1% dithicthreitol (DTT) (1). As described in last years report, toxin A is produced in equal amounts on TSB_D and in TSB_D + NTA whereas S production increases when the bacteria are grown in the presence of NTA. Furthermore, the enzymatic activity of A increases with preincubation in 4M urea and 1% DTT whereas S activity is reduced by such treatment (1). The toxin phenotype of potential S producers was then verified by preincubating culture supernatants in specific anti A or anti S serum. The production of these antisera were described in last years progress report.

3. Animal Models. The virulence of strain Ps 388 and a protease deficient mutant Ps 388-6 was tested in the mouse eye model; bacteremia in agranulocytic rabbits; and vasculitis in agranulocytic rabbits. Strains Ps 388 and Ps 388-6 produce exoenzyme S but not toxin A.

The *P. aeruginosa* eye model was that described by Hazlett et. al. (3). Groups of six mice each had their corneas slightly wounded (3) then 10µl containing from 10^4-10^8 bacteria were dropped on the right wounded cornea. Sterile culture medium (m0ml) was dropped on the left wounded cornea which served as a control. The animal's corneas were observed microscopically every other day to determine the extent of gross damage. Animals were scored using a corneal damage index (4) of 0=normal through +4 (4=perforated cornea). Experiments were terminated after 30 days. In past experiments we observed that the eye damage did not change after 16 days post infection. Infections caused by Ps 388 and Ps 388-6 were compared to those caused by the toxin A

positive, S negative strain PA103 and the toxin A negative S negative strain WR-5. These strains have been previously characterized (5).

The rabbit models were those developed by Ziegler and Douglas (6) and these experiments were done in collaboration with Dr. E. Ziegler, U. Calfornia at San Diego. Each group consisted of 7-8 rabbits. The rabbits were injected intravenously with nitrogen mustard mechlorethamine (Merck, Sharp and Dohme, West Point, PA) in a dose of 3.0 ng/kg. Seventy two hours later 1-7 X 10^7 bacteria in 0.1 ml of TSB_D broth were instilled into the conjunctival sac of the right eye. Animals were observed for one week for signs of illness. After 7 days or immediately after animals died, blood was drawn aseptically from the heart and 1.0ml mixed with 9.0ml melted TSA. Plates were read after 24 hr. incubation at 37°C. Histological sections were prepared from representative blood vessels, stained with hematoxylin-eosin and observed microscopically. In addition to stain Ps 388, Ps 388-6, WR + 5 and PA103, strain #3 previously described (6), was used as a positive control.

In order to determine if Ps 388 and Ps 388-6 could induce any vasculitis, rabbits (6 per group) were injected with nitrogen mustard as described above and 72 hrs. later 1 X 10^7 bacteria in 0.1ml TSB_D were injected intradermally. Skin lesions were observed for 3 days and 2 animals from each group sacrificed 24, 28, and 72 hours after bacterial injections. Histological sections were made from tissue surrounding the site of injection, stained and observed as described above.

C. Results.

1. The effects of various chelators on toxin S production by strain Ps 388. Previously, we observed that S production by strain Ps 388 required the presence of NTA and that 10mM NTA gave maximum S yields. We have extended these results by comparing the effects of various chelators on bacterial growth and S yields. The chelating agents tested were tricine, citrate and ethylenediaminetitroacetate (EDTA). As shown in Table 1, the addition of (0.1mM-20mM) tricine or citrate to the medium in place of NTA resulted in negligible S yields. The addition of EDTA (10-20mM) to the medium resulted in S yields equal to the yields obtained when 10mM NTA was present (Table 1 and figure 1 of last years progress report). However, EDTA at 10-20mM, inhibited bacterial growth and pigment production by strain 388. Therefore, we routinely use 10mM NTA to supplement TSB_D for S production. A manuscript containing our initial studies on factors that influence the production of S is currently being prepared. A copy of a draft of that manuscript is appended as paper #2 in the appendix.

2. Production of S in vivo by strain 388. Last year we reported on the production of S in burned mice infected with strain Ps 388 (see progress report 1978). We have now extended our observations on the specificity of the enzymatic activity detected in the sera and skin extracts of Ps 388 infected burned mice. As described above (section IB2) the enzymatic activity of S is decreased by incubation of the enzyme with 4M urea and 1% DTT. The treatment of skin extracts or sera from burned Ps 388 infected mice with urea and DTT partially destroyed their enzymatic activity (Table 2). To determine if the skin extracts or sera contained a factor which might alter these enzymes, crude S or crude toxinA was preincubated in skin extracts or sera from burned

Table 1. Effect of metal chelators on bacterial growth and exoenzyme S yields in cultures of P. aeruginosa 388[a]

Conc. of chelator	Tricine		Citrate		EDTA	
	Bacterial growth	ADPR incorporated	Bacterial growth	ADPR incorporated	Bacterial growth	ADPR incorporated
mM	(OD_{540})	(pmoles)	(OD_{540})	(pmoles)	(OD_{540})	(pmoles)
0.1	8.4	1.0	9.0	0.6	8.4	0.5
1.0	8.7	0.6	9.0	0.4	8.4	0.6
10.0	8.4	0.7	8.7	0.6	6.0	667
20.0	8.4	0.7	9.0	0.4	5.7	620

[a] Media were supplemented with 100mM MSG and 1% glycerol. Cultures were incubated for 22 h with shaking at 32°C.

Table 2. Effect of urea and dithiothreitol on the ADP-ribosyl transferase activity of mouse skin extracts and sera

	ADPR incorporated (pmole)[a]	
	$+H_2O$	+urea, DTT
A. Control skin extracts or sera + toxin A or exoenzyme S[b]		
Skin extract + toxin A	0.8	12.4
Skin extract + exoenzyme S	8.5	4.9
Serum + toxin A	1.7	13.4
Serum + exoenzyme S	11.9	5.5
B. Skin extracts from burned infected mice		
Skin no.		
3	28.0	15.4
23	48.1	32.1
37	60.0	45.9
39	50.9	29.0
56	25.9	19.6
C. Sera of burned infected mice		
Serum no.		
19	14.3	6.8
21	13.3	8.2
23	9.5	6.5
37	26.7	17.0
41	12.1	4.5

[a] Per 10 µl of skin extract or serum.

[b] Crude toxin A (10 µl) or crude exoenzyme S (10 µl of a 1:10 dilution) were pre-incubated with 90 µl of skin extract or sera for 1 h at 37°C before being tested for ADP-ribosyl transferase activity.

non-infected mice at 25 C for 15 min. before assaying their enzymatic activities. The preincubation of these enzymes in uninfected mouse sera or skin extracts did not alter the enzymatic properties of crude S or A (see Table 2 controls). We have also measured the active EF-2 content of liver, kidney and spleen cells from Ps 388 infected burned mice and compared these to levels in tissues from uninfected or PA103 infected burned mice (Table 3). In agreement with previous reports (5,7,8) the levels of active EF-2 in tissue from burned mice infected with strain PA103 (toxin A^+S^-) were markedly decreased at 24 hr. postinfection (Table 3). In contrast, active EF-2 levels in burned mice infected with strain in 388 were normal in the livers, kidneys and spleen at 24 hr post-infection (Table 3). Thus strain Ps 388 does not produce detectable toxin A in vivo and the S that is produced while detectable in serum and skin extracts, does not result in altered EF-2 levels. These studies and those discribed in detail in last years progress report have now been published (9). A copy of this paper is appended (paper #1).

3. Production of Toxin S by Clinical Isolates of P. aeruginosa. Utilizing the methods described above (section IB 2) we have initiated a study to determine the incidence of toxin S production by strains of P. aeruginosa isolated from patients having bacteremia. These methods permit us to distinguish between strains that produce toxin A but not S (A^+S^-); those that produce both toxins A and S (A^+S^+); those that produce S but not A (A^-S^+) and those that do not produce either A or S (A^-S^-). A total of 39 strains have thus far been examined (Table 4). Twenty five (64%) were A^+S^-; 4 (10%) were A^+S^+, 3 (8%) were A^-S^+ and 7 (18%) produced no detectable enzymatic activity (A^-S^-). Of the 7 strains identified as producing S toxin, 5 were from patients that died. Thus while the overall mortality rate of these bacteremic patients was 43% the mortality rate of those individuals infected with toxin S producing strains was 71%. This increased mortality rate was not associated with the toxin A phenotype (Table 4). We plan to continue this study since the numbers of strains tested are small.

4. Virulence of A^-S^+ strains in various animal models. Previous studies (9) in last years progress report showed that Ps 388 that produces toxin S but not A (A^-S^+) was virulent in burned mice. This year we tested the virulence of Ps 388 and the protease deficient mutant Ps 388-6 in 3 additional animal models as described above (IB 3).

Strains Ps 388 and Ps 388-6 were avirulent in the mouse eye model. Thus even at the highest inocula tested, (10^8) these strains failed to produce any lasting damage to the mouse eyes. In contrast the toxin A^+S^- strain PA103 produces severe corneal damage (CDI>3) with an inocula as small as 10^6 (Fig. 1).

Strains Ps 388 and Ps 388-6 were also avirulent when instilled into the intact conjunctival sac of agranulocytic rabbits (Table 5). Thus neither Ps 388 or Ps 388-6 caused mortality or vasculitis in these rabbits. Strain WR-5 (toxin A^-S^-) was also avirulent in this model and strain PA103 (toxin A^+S^-) was of low virulence. By contrast inoculation of strain 3 (6) resulted in 100% mortality and 100% vasculitis (Table 5).

While these results may indicate that S is not important in these two animal models it seems equally possible that strains Ps 388 and Ps 388-6 lack some other required virulence factor. Thus Ps 388 was highly virulant

Table 3. Comparison of the active EF-2 levels in tissue extracts from burned mice infected with *P. aeruginosa* PA-103 or 388[a]

	% Control active EF-2 levels[b]	
Organ[c]	PA-103 infection	388 infection
Liver	35	102
Kidney	83	95
Spleen	82	101

[a] Mice were sacrificed 24 h after being burned and infected.

[b] Control values were obtained using the appropriate tissue from anesthetized and burned uninfected mice.

[c] Organs from six similarly treated mice were pooled.

Table 4. Incidence of Toxin S Production by P. aeruginosa isolated from patients with bacteremia.

Toxin Phenotype	Non-Fatal Bacteremia	Fatal Bacteremia	All Isolates
A^+S^-	16(73%)	9(52%)	25(64%)
A^+S^+	1(4.5%)	3(18%)	4(10%)
A^-S^+	1(4.5%)	2(12%)	3(8%)
A^-S^-	4(18%)	3(18%)	7(18%)
	22(100%)	17(100%)	39(100%)

Figure 1

Average corneal damage index in mouse eyes 22 days after infection with strain PA103 (o--o), Ps 388 (●—●) and WR-5 (▲—▲).

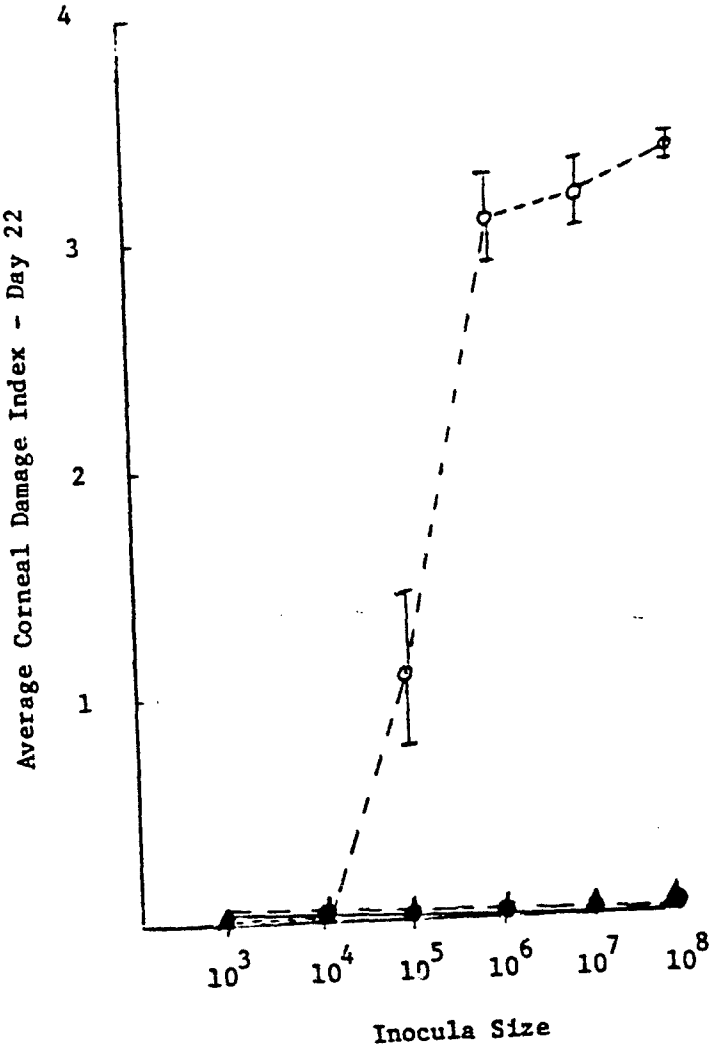


Table 5. Virulence of various P. aeruginosa strains in neutropenic rabbits following conjunctival inoculation.

Strain	Eye Inoculum (CFU)	Mortality	Vasculitis
Ps 388-0	3×10^7	0/8	0/8
Ps 388-6	2×10^7	0/8	0/8
PA 103	1×10^7	0/7	2/7
WR-5	7×10^7	0/8	0/8
#3	3×10^7	8/8	8/8

in the burned mouse model (see 1978 progress report) and both Ps 388 and Ps 388-6 caused vasculitis when 2 X 10^7 bacteria were injected intradermally into neutropenic rabbits (see method section IB 3). Microscopic sections showed that all the blood vessels, both arterial and venous, in the area of the injection, were surrounded by a thick sheath of hematoxylin-positive material composed entirely of bacilli. This was observed with 100% of the injected animals.

D. Discussion

We have completed our initial studies on the production of S in vitro and in vivo. Conditions have been defined for production of large amounts of S in vitro. We have demonstrated that S is produced in vivo and have established assays for distinguishing between the enzymatic activity of S and A. Preliminary results indicate that bacteremic patients infected with S producing strains have a higher mortality rate than those infected with A^+S^- or A^-S^- strains of P. aeruginosa. Additional strains need to be examined to verify this conclusion.

While Ps 388 (toxin A^-S^+) strain is virulent in the burned mouse model or when injected intradermally into neutropenic rabbits it is avirulent in the mouse corneal model and when it is instilled into the intact conjunctival sac of neutropenic rabbits. Thus it appears Ps 388 may lack a factor required for invasion. The additional toxin A^-S^+ strains isolated from patients with fatal bacteremia will be compared to Ps 388 and tested to see if they are virulent in these various animal models.

II. Assay Conditions for Measuring the Enzymatic Activity of Toxin S.

A. Introduction

The optimum assay conditions for the enzymatic activity of diphtheria toxin fragment A were determined a number of years ago and a very similar assay was found to be ideal for P. aeruginosa toxin A (2). When toxin S was discovered, the same assay was employed for it as for toxin A (1). However, some exploratory work showed these conditions were not ideal for quantitating S enzymatic activity. Thus we have examined a number of parameters to determine the optimum conditions for assaying the enzymatic activity of toxin S.

B. Methods

1. Substrates. Wheat germ extract was prepared as previously described in detail (2) using phenylmethylsulfonyl flouride (PMSF), reducing agents and a hypotonic buffer. In spite of the presence of PMSF these wheat germ extracts have relatively high protease activity, therefore we also used rat liver as a source of substrate protein(s) for toxin S. Rat liver was extracted using 0.25M sucrose containing PMSF but no added reducing agents. The 100,000 X g supernatant of the liver extract was desalted on a Sephadex G-25 column into 10mM Tris, pH 7.0 and frozen in small aliquots at 70°C.

2. Assay Conditions. The optimum assay conditions for toxin S were developed using the soluble rat liver extract. However, there was little relative difference seen between the wheat germ and rat liver extracts under many different assay conditions. The advantages to using rat liver are that it is a well understood system and, when extracted with PMSF it is relatively free of protease activity. The standard assay for toxin S was as follows: 50 µl of buffer, 10 µl of desalted liver extract, a variable amount of diluted crude toxin S (2-10 µl), 5 µl of ^{14}C-labeled NAD, and incubation at 25°C for a set period of time. Crude and desalted crude toxin gave the same results whenever compared.

3. Buffers tested. Below is a list of buffers tested. For comparison, all buffers were used at 50mM concentration and the pH was adjusted with NaOH, KOH, or HCl.

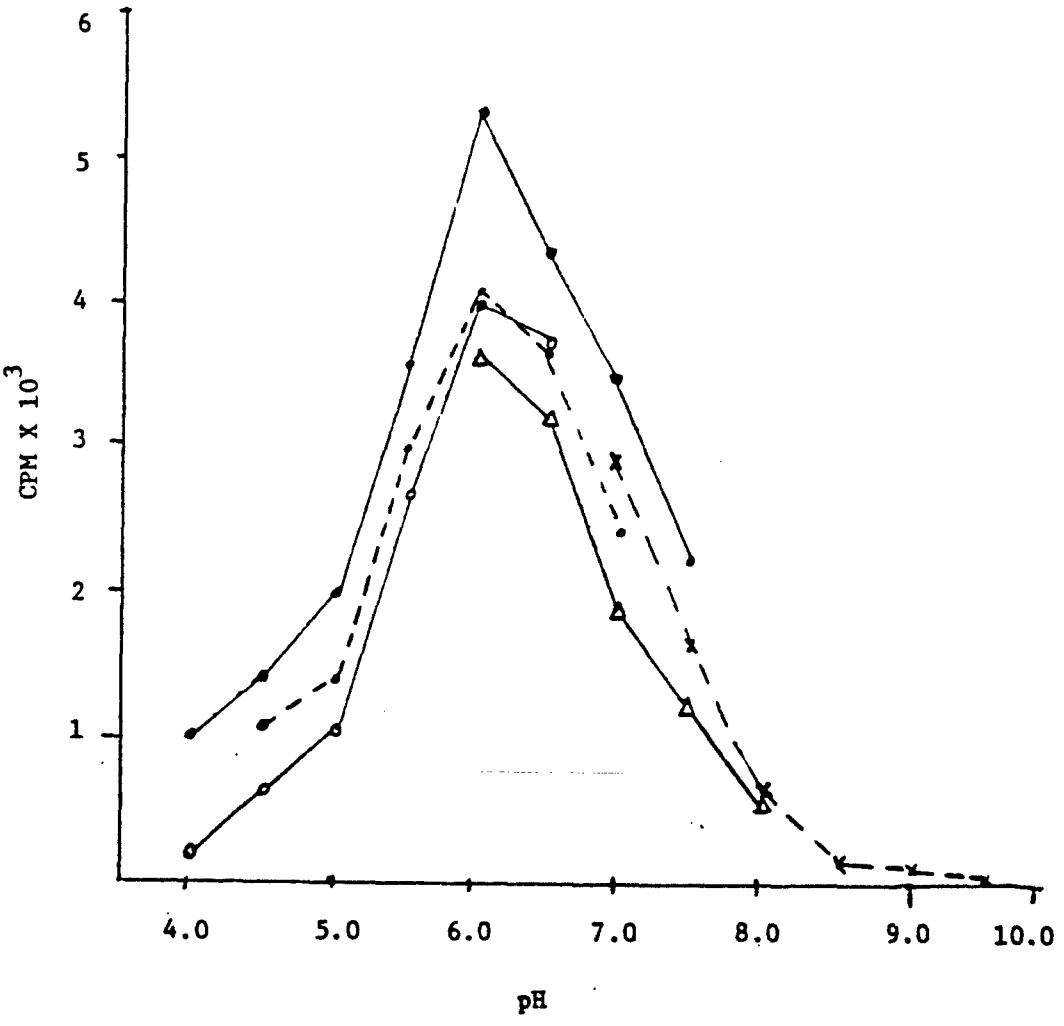
Buffer	pH range
N-2-acetamidoiminodiacetic acid (ADA)	4.0-7.5
acetic acid	4.0-6.5
2-(N-morpholino) ethanesulfonic acid (MES)	4.5-7.0
histamine	4.5-7.0
histidine	4.5-7.0
imidazole	6.0-8.5
sodium or potassium phosphate	6.0-8.0
N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES)	6.5-8.5
tris	7.0-9.5
sodium borate	7.5-9.5
sodium bicarbonate	8.0-10.0

C. Results

1. Effect of pH and ionic strength on enzymatic activity of toxin S. In all buffers tested, the maximum enzymatic activity with toxin S occurred at pH 6.0. At pH 7.0 there was still about 60% of the activity while at pH 5.0 the activity dropped to about 35% (fig. 2). A pH optimum of 6 for toxin S is quite different than that for diphtheria toxin fragment A or Pseudomonas toxin A which have an optimum pH of 8.0 (10).

Figure 2

Effect of pH on the enzymatic activity of toxin S in rat liver extracts. Buffers used include; 50mM ADA (●—●), 50mM Histidine (●---●), 50mM acetate (○—○), 50mM $NaHPO_4$ (△—△) and 50mM tris (X---X).



Using desalted liver extract and desalted crude toxin S, we investigated the effect of ionic strengh on enzymatic activity. The activity in the region from pH 5-7 was insensitive to ionic strength but the activity in the region above pH 7.0 was rather sensitive to salt concentrations. At pH 8.0 with 50mM imidazole; increasing the concentration of NaCl or KCl from 0 to 100mM markedly increased the enzymatic activity. At concentrations above 100mM, NaCl or KCl, were slightly inhibitory (Table 6).

The buffers tested gave nearly the same activity when the pH and ionic strength were accounted for except ADA, in which case, the activity was about 20% higher. Sodium borate almost completely inhibited toxin S activity probably due to the well known ability of alkaline borate to complex with cis-hydroxyl groups such as those on ribosomes.

2. Effects of Metals and Detergents on the enzymatic activity of toxin S. The following metals; Fe^{+3}, Cu^{+2}, Cn^{+2}, Mn^{+2}, Mg^{+2} or Zn^{+2}, were added to the enzyme assay at a final concentration of 0, 10 or 100mM. The addition of these metals did not increase enzymatic activity, therefore there is no apparent requirement for these metals. However, Fe^{+3} and Cu^{+2} actually had an inhibitory effect on the enzymatic activity of toxin S (Table 7). This inhibition could be seen when as little as 1μM $FeCl_3$ or $CuSO_4$ was added to the assay and was greatest at a concentration of 30μM $FeCl_3$ or $CuSO_4$. When the concentration of these metals was increased to 100mM this inhibition was again minimal. We are continuing to investigate this effect to determine if these two metals are affecting the substrate or the enzyme (s) and to determine why the inhibition is abolished at very high metal concentrations.

The enzymatic activity of crude toxin S was not inhibited by preincubation with 1% SDS or 1% triton X-100. However, the enzymatic activity was completely inhibited by the addition of as little as 0.03% SDS or 1% triton X-100 to the assay reaction mixture. This suggests that these detergents are either reversably affecting toxin S or adversely affecting the substrate.

D. Discussion

The studies described above provides further evidence that the ADP-ribosyl transferase activity asscciated with toxin S is different from any previously described. Thus the pH optimum for S is 6 whereas all other ADP-ribosyl transferases have alkaline pH optimum (11); there is no metal requirement and very low concentrations of $FeCl_3$ or $CuSO_4$ markedly inhibit S activity whereas substantially higher concentrations of iron are required to inhibit toxin A activity (12). As indicated above, some of these studies will be continued this year. The results of these studies should elucidate tne optimum conditions for assaying the enzymatic activity of S which will facilitate monitoring the purification of S and its substrate.

III. Purification and Characterization of Toxin S.

A. Introduction: We have continued our studies on the purification and characterization of toxin S produced by strain Ps 388-6. We have compared

Table 6. Effect of salt concentration on the enzymatic activity of toxin S.

NaCl conc	Relative Activity[1]
0	10
10mM	20
20mM	45
50mM	90
100mM	100
200mM	95
500mM	80

[1] The enzyme assays were run for 15 min. at 25°C with 50mM imidazole pH 8.0.

Table 7. Effect of Iron and Copper on the enzymatic activity of toxin S.

Metal	Conc.	% Relative Enzymatic Activity
none	—	100
$FeCl_3$	1μM	97
	3μM	84
	10μM	46
	30μM	11
	100μM	81
none	—	100
$CuSO_4$	1μM	95
	3μM	79
	10μM	50
	30μM	15
	100μM	93

two purification schemes based on conventional protein chemistry methods and have initiated studies on the use of detergents to solubilize and stabilize toxin S. We have further characterized S by comparing the two enzymatically active peptides with approximate molecular weights of 30K and 60K daltons with respect to substrate specificity, reaction with antisera and molecular weight.

B. Methods

The following list of methods do not include those presented in the previous annual report; they reflect modifications of old or entirely new procedures.

1. Production of toxin S in large batches. The procedure used for 16L fermenter batches is presented in the progress report for 1977-1978 with modifications as follows: Chelex-100 (BioRad, Richmond, Ca) was added to media as a 10X concentrate prior to dialysis. (10g Chelex/L final media volume). Cultures were harvested at 15-16 hours. A 20L capacity microferm fermenter (New Brunswick Scientific, New Brunswick, NJ) was used to prepare 16L batches of Ps 388-6.

2. ADP-ribosyl transferase acticity. The ADP-ribosyl transferase assay has been modified so that when it is not necessary to monitor enzyme activities for specific activity (ie. in following toxin on gel electrophoresis, column chromatography or other purification steps) we use less isotope and wheat germ extract and if necessary run the reaction for longer than the conventional 5 min. In monitoring column elution we use 10µl wheat ger extract, 10µl water(or sample)and 2µl NAD^+ thereby significantly reducing the cost per assay.

3. Protein Determination. Protein concentrations were determined by the method of Bradford (13) modified by using a commerical reagent (Bio-rad Protein assay dye reagent concentrate) purchased from Bio Rad Lab, Richmond, California. Bovine gamma globulin (Bio-Rad) was used as the standard.

4. Autoradiography. Autoradiography of ^{14}C labeled proteins separated in polyacrylamide gels was done using the method of Bonner and Laskey (14). Following electrophoresis the gel was fixed in 12.5% TCA overnight then washed two times with dimethyl sulphoxide (DMSO), 300ml/gel, for 30 min. This gel was then soaked in a solution of 2,5-diphenyloxazole (PPO) in DMSO, dried and exposed to RP Royal "X-Omat" film at $-70°C$.

C. Results

Frozen post DE-52 partially purified toxin S obtained from the second and third fermenter run (annual report, 1978) was used as a starting point for the next steps in purification. Since enzymatic activity as followed by the ADPR ribosyl-transferase assay elutes in the void volume of

both G-100 sephadex and Biogel A.5M columns and does not penetrate a non denaturing 6% polyacrylamide gel under electrophoresis (ie. demonstrating aggregation) it was decided to investigate other procedures relying on seperation by charge, rather than molecular weight. Methods attempted included preperative isoelectric focusing, binding and gradient elution from DEAE-sephadex and cellulose, and binding followed by gradient elution from hydroxyapatite (HA). Binding followed by gradient elution from hydroxyapatite gives better resolution and material of higher specific activity than either of the other procedures. Binding and elution of S from hydroxyapatite was found to be variable with respect to whether sodium or potassium phosphate buffer was used. With sodium phosphate, virtually all the enzyme activity bound was eluted between 20-100mM phosphate at pH 6.8. With the potassium salt, elution occurs at 400-700mM KPO_4. Since very little protein was found to bind to hydroxyapatite at low phosphate concentration (25mM) but most of the toxin activity did bind, it was decided to try using HA as a first step in purification. It was reasoned that if binding was specific, then the cumbersome DE-52 batch process (requiring up to 1.5 kg new DE-52 and the processing of 64L of diluted culture supernatant) might be replaced with a single batch binding of toxin to hydroxyapatite, then batch or gradient elution.

Initial binding studies with small volumes of culture supernatants indicated that it is indeed possible to bind up to 95% of the ADP ribosyl transferase activity in 1.5-4 hours. Binding is optimal with supernatant diluted 1:1 with water, and a reducing agent present (2me or DTT at 5-10mM). Binding was found to vary depending on the batch and quality of hydroxyapatite used. Fractured hydroxyapatite (useless for column chromatography) was found useable in batch preparations since it not only may be removed from samples with relatively low centrifugal force, but also was found very efficient at binding toxin. Thus in the next two fermenter runs, F IV and F V, toxin was bound directly to HA. Although purification in this one step procedure approaches 15 fold, binding efficiency was drastically reduced in fermenter scale binding (only 50% of the enzyme recoverable from culture supernatant bound to the HA (5 g/100ml supernatant) after 8 hours). It was determined that half the recoverable bound activity was in the hydroxyapatite fines which must be removed by centrifugation and half was in the HA that settled out in two hours time (approximately 90% of the total HA). Mixing of HA in the 32 liter volume of dilute culture supernatant was by paddle in both fermenter runs and fresh (low in fines) HA was also used. Therefore, as performed, the total recovery of enzyme from the F IV and F V fermenter runs was relatively low compared to the 80% recovered from F II (approximately 30% and 20% respectively). However, the material eluting from the HA is of greater specific activity in enzyme assay compared with batch binding/elution with DEAE cellulose (Table 8).

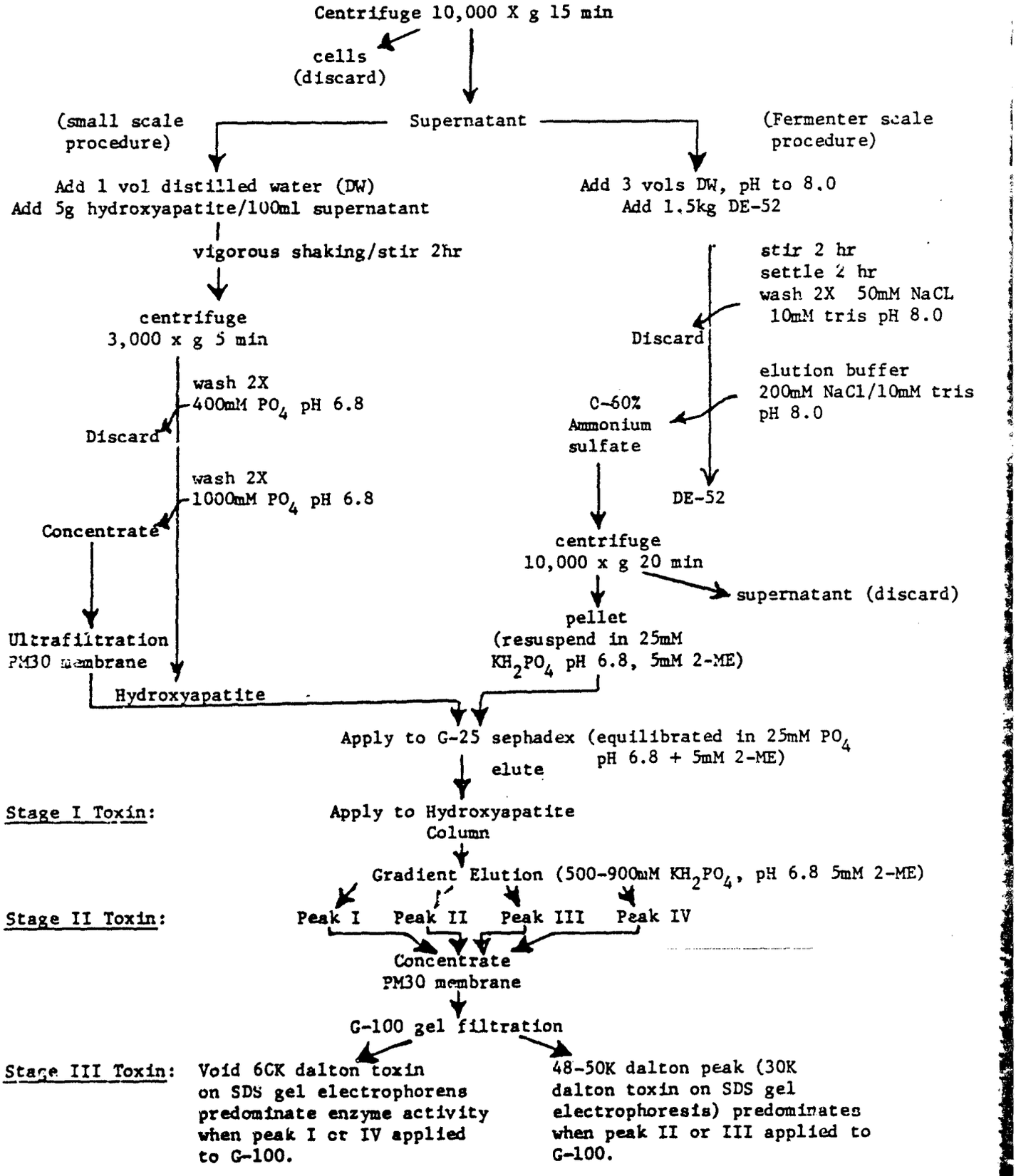
Since prior work with post DEAE-cellulose toxin has shown the feasability of using HA gradient elution for further purification, we decided to divide the F IV fermenter post HA (stage I material in Figure 3) into 5 aliquots for the purpose of trying a second step of binding to HA with phosphate gradient elution. An optimum separation was obtained by 1) G-25 gel filtration into 25mM potassium phosphate pH 6.8 to remove excess phosphate from

Table 8. Toxin S activity: comparison of 16L fermenter run II (DE-52 batch preparation) and fermenter V (hydroxyapatite batch purification).

Material	Protein g	Toxin Activity[1] recovered %	Purification fold
FII Cultural supernatant	4.8	100	1
Stage I: post 0.15M NaCl wash	0.48	60	6.0
: post 0.25M NaCl wash	0.88	20	1.7
FV Culture supernatant	12.5	100	1
Stage I: post hydroxyapatite post G-25	0.28	18	8

[1] All enzyme activity was bound to DE-52 in the F II batch. 50% of the measureable toxin activity in F V was bound to hydroxyapatite; 18% of the activity bound was eluted. Hence recovery of toxin bound to hyroxyapatite was 35%. Electrophoretic analysis of post hydroxyapatite toxin demonstrates that the preparation has fewer contaminating proteins that the post DE-52 material (~25 fold vs 6 fold). However, the specific activity of the toxin does not rise proportionally, indicating enzymatic inactivation.

Figure 3 Partial purification scheme for Toxin S from Pseudomonas aeruginosa 388-6.



batch preparation, 2) binding to a previously equilibrated HA column at 25mM potassium phosphate pH 6.8, then 3) elution with a 500-1000mM linear gradient of phosphate (fig. 4). All buffers were made 5mM with DTT or 2-ME. Four regions of enzyme activity occur, at approximately (peak I)520, (peak II)600, (peak III)630mM, and in a broad region between 700-800mM (peak IV). Approximately 60% of the protein bound to the column eluted in these peaks; however, 70% of the enzyme activity was found in peak III corresponding to approximately one fourth the applied protein. Typical specific activities of these elutes are listed in Table 9. The peak with the highest specific activity (peak III) was further characterized on denaturing and non denaturing polyacrylamide gel electrophoresis (fig. 5,6) and with G-100 sephadex chromatography (fig. 7). Also, the reaction products of this toxin in wheat germ extracts were compared with those observed with crude S by autoradiography of SDS-polyacrylamide gels run on toxin-wheat germ reactions products.

Toxin eluted from the batch hydroxyapatite treatment of culture supernatant, like that observed with DE-52 batch treatment, is seperable on SDS-polyacrylamide gel electrophoresis into enzymatically active material with molecular weights of approximately 60K and 30K daltons. After seperation of toxin activities on the hydroxyapatite gradient, a difference is observed in the relative abundance of 30K dalton enzyme and 60K dalton enzyme. Upon SDS gel electrophoresis, peaks II and III demonstrate most of their eluteable enzyme activity at 30K (based on protein standard calibration of the SDS gels). Under these conditions, the I and IV regions demonstrate most of their activity at 60K daltons. Gel filtration of peak I under non denaturing conditions however demonstrates that enzyme activity elutes primarily in the void volume, with some elution at 50K as well (Fig. 8).

Electrophoresis in nondenaturing 7½% polyacrylamide gels of peak III toxin demonstrates seperation of toxin into three major peaks of activity corresponding to major protein band on a similar stained gel. G-100 sephadex chromatography of the same peak III toxin demonstrates a single peak in enzymatic activity coincident with OD_{280} at about 50K daltons, with no activity eluting in the void volume, based upon previous column calibration with protein standards. Hence under non denaturing conditions, some post hydroxyapatite gradient material (peak I) behaves predominately as crude S and aggregates to a complex with a size of >100K daltons, and some (e.g. peak III) elutes predominatly as a single peak at 50K. SDS gel electrophoresis then demonstrates that most 60K dalton material will elute in the void volume;enzyme activity from a G-100 column (corresponding to 60K toxin in SDS electrophoresis) is neutralizable by anti S antibody, the 50K (corresponding to 30K daltons in SDS electrophoresis) is not neutralized by either anti A or anti S antisera (Table 10). Neutralization of toxin eluted from analytical SDS polyacrylamide gels (60 or 30K dalton) has not been shown with either anti A or anti S perhaps because SDS may interfere with complex formation.

Autoradiography of reaction products of toxin S using wheat germ extracts as substrate demonstrate that toxins that are electrophoretically purified (ie. toxin eluted from SDS polyacrylamide gel slices with molecular weights corresponding to 60K and 30K daltons) both ADP-ribosylate primarily

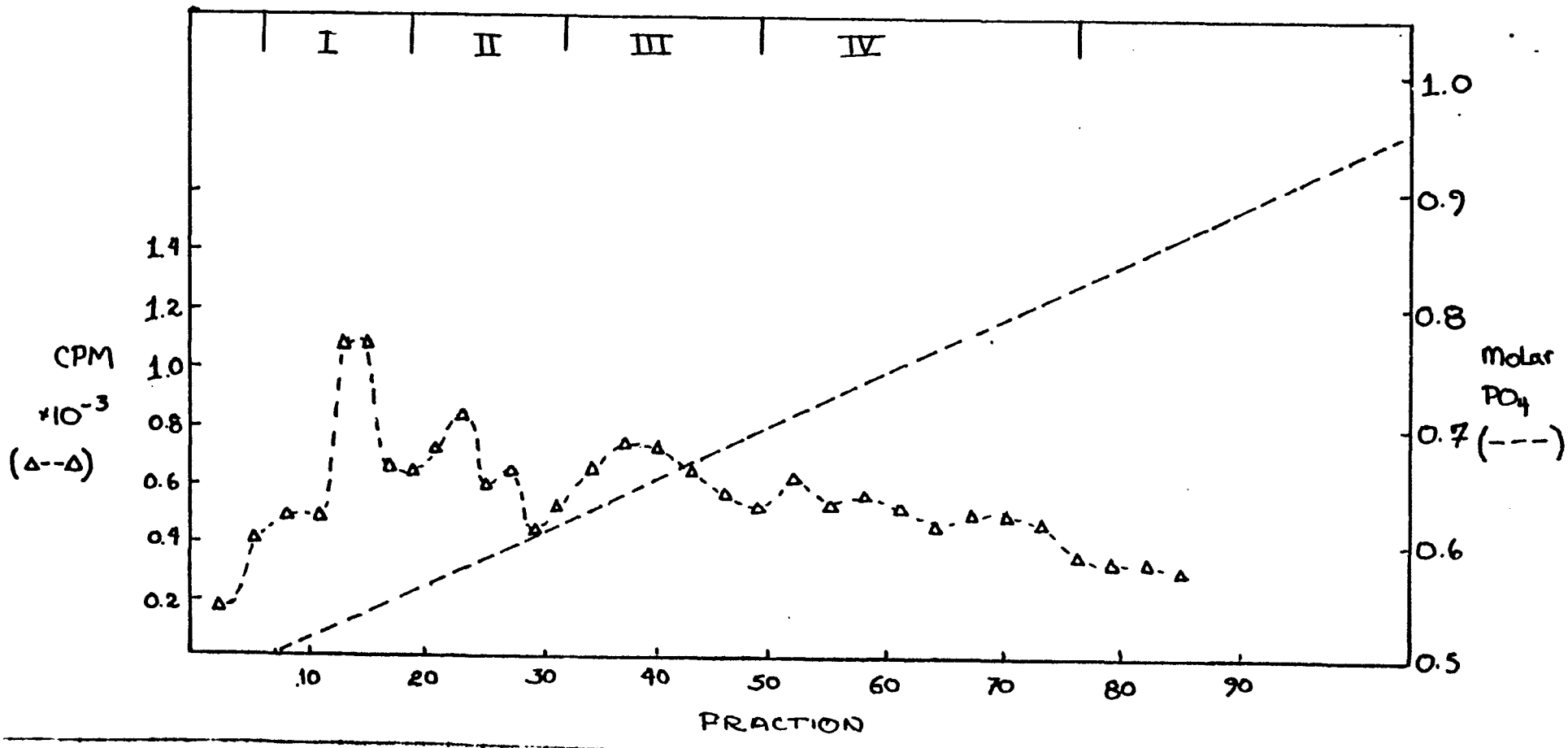


Figure 4 Hydroxyapatite gradient elution of Toxin S.

30mg of toxin S eluted from batch treatment (stage I) hydroxyapatite and equilibrated with 25mM potassium phosphate pH 6.8, 5mM DTT by gel filtration with sephadex G-25, was applied to a 28ml column of equilibrated hydroxyapatite. A 25ml wash of 500mM PO_4 was applied immediately thereafter, then a 500mM-1,000mM PO_4 gradient was started (500ml). Fractions were assayed for enzymatic activity and pooled accordingly in to four fractions pooled fractions were concentrated by Amicon PM-30 ultrafiltration, reequilibrated into 25mM PO_4 pH 6.8 plus 5mM DTT by G-25 sephadex chromatography, then frozen an -70°.

Table 9. Specific activity of partially purified toxin S preparations, Fermenter IV (Hydroxyapatite batch purification).

Material	Incorporation[1] pmoles/ng protein/5 min @ 25°C
388-6 culture supernatant	0.3
Stage I toxin	0.6
Stage II toxin	
hydroxyapatite peak I	0.4
hydroxyapatite peak II	1.1
hydroxyapatite peak III	1.6-2.5
hydroxyapatite peak IV	0.45

[1] Reaction conditions are standard, 44ng of toxin protein is used in each assay (Bradford portein determination).

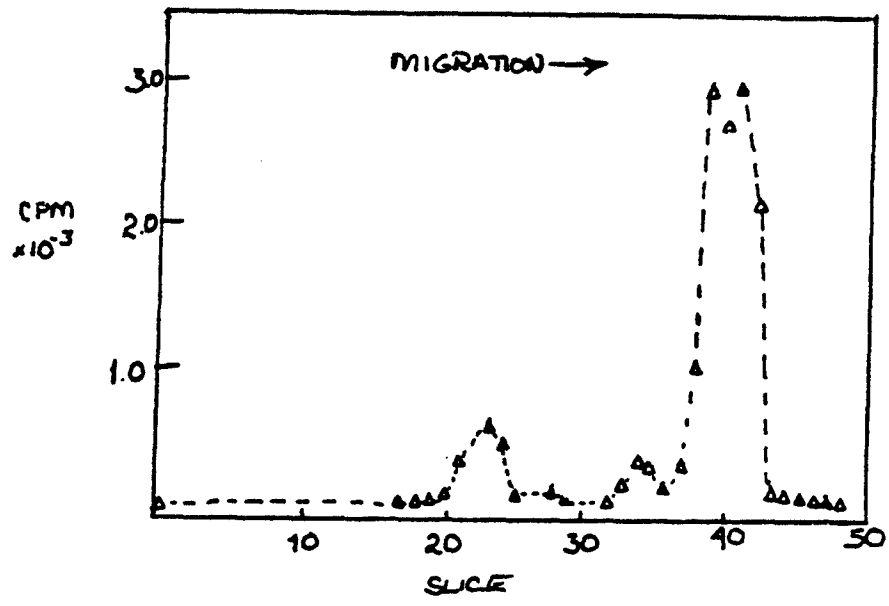


Figure 5

25µl of hydroxyapatite gradient peak III was heated for 30 min. at 37°C with sample solution consisting of SDS, 2-mercaptoethanol and tris buffer, pH 6.8. This was electrophoresed in a 12% gel. Corresponding gels were stained for protein or sliced for enzymatic activity and neutralization studies. Slices were eluted into 10mM tris pH 7.0 plus 10mM DTT.

Figure 6 Non-SDS gel electrophoresis of Toxin S.

50µg of hydroxyapatite gradient peak III was applied to 7.5% cylindrical gels. Gels were stained for protein or sliced. Slices were eluted into 150µl 10mM tris pH 7.0 plus 10mM DTT overnight @ 4°C. 1.5µl was assayed for enzymatic activity under standard conditions. Toxin activity is associated with each protein staining band.

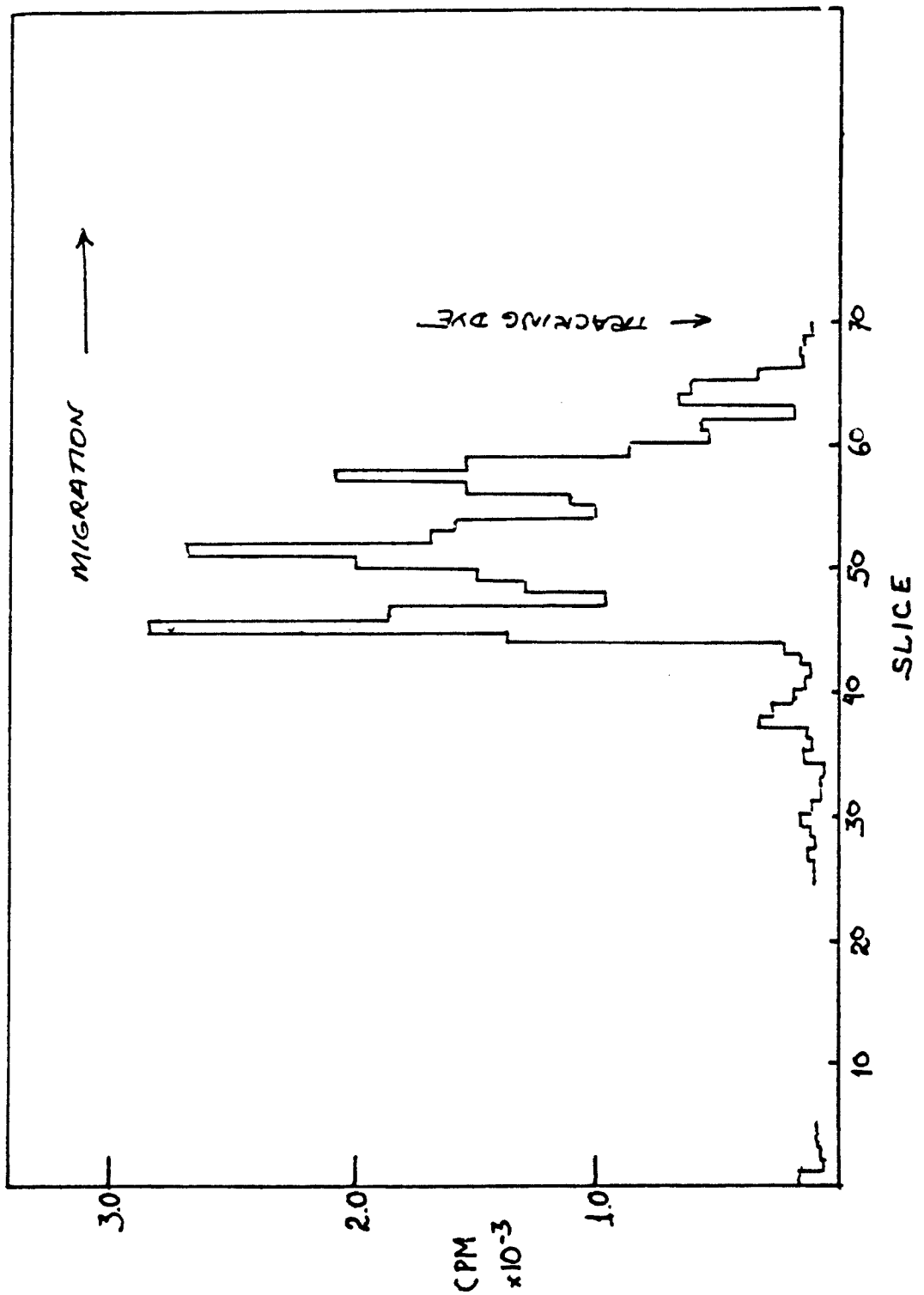


Figure 7 G-100 sephadex chromatography of Toxin S.

Approximately 1mg hydroxyapatite gradient peak III eluate in 50µl total volume was applied to a 0.5 X 26cm column. 200µl fractions were assayed for enzymatic activity. 1.5µl of 1:20 dilutions of each fraction were assayed under standard conditions. OD_{280} was obtained with in line monitoring. The column and sample was equilibrated in 25mM potassium phosphate pH 6.8 buffer with 10mM DTT. The column was previously calibrated with protein standards. Fraction 21 corresponds to a molecular weight of 48K daltons by least squares analysis.

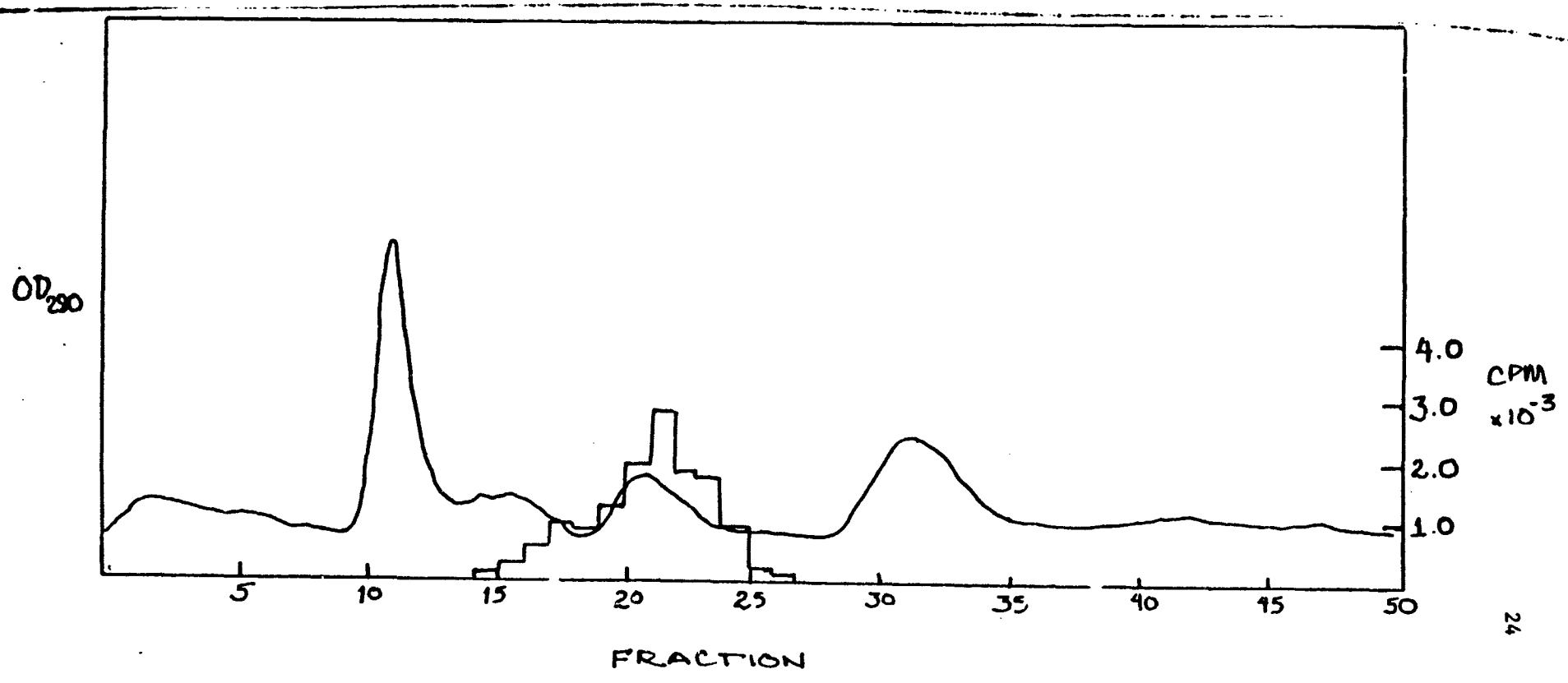
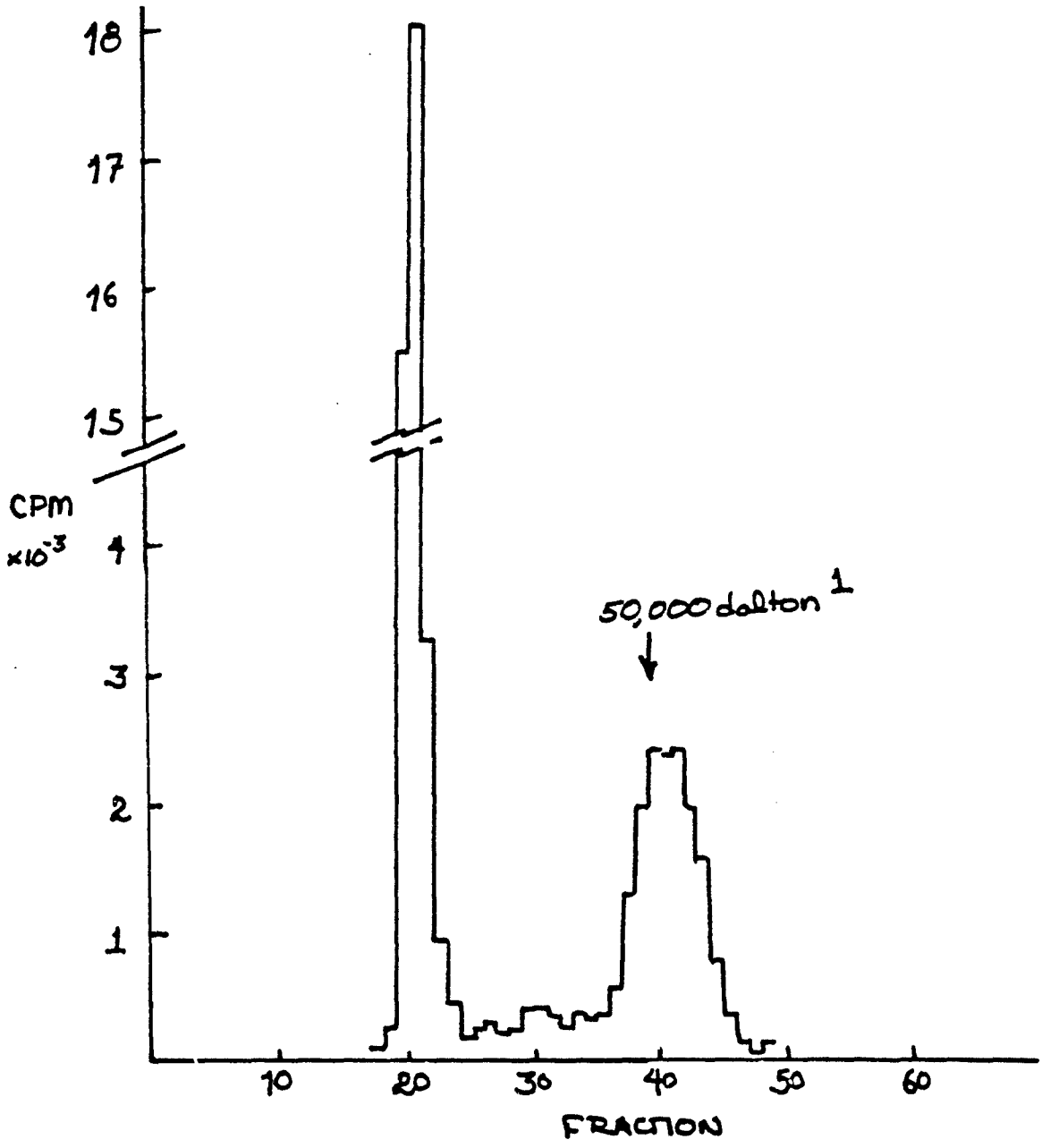


Figure 8

G-100 sephadex elution profile of enzymatic activity from HA gradient peak IV.



[1] The gel filtration column was calibrated for molecular size with protein standards (Linear Regression R^2 = .98)
The 1.1 X 55cm column was equilibrated in 25mM potassium phosphate pH 6.8 5mM 2-ME. Sample was applied in 200µl. 1.5ml (approximate volume) fractious were obtained and 5µl of each fraction (or dilution thereof) was assayed under standard conditions for ADP-ribosyl transferase activity. Peak fractions were pooled and assayed for neutralizability by toxin A and toxin S anti serum.

Table 10. The effect of anti toxin A and anti S immune sera on ADP-ribosylation reaction.[1]

Material[2]	Counts per Minute		
	NRS[3]	Anti A[3]	Anti S[3]
Toxin A	439	520	126
Toxin S culture supernatant	646	92	827
Toxin S G-100 void peak	480	445	140
Toxin S G-100 50K dalton peak	1,763	1,958	1,640

[1] Samples of diluted toxin in 10µl were preincubated with 10µl serum for 15 min. at 37°C then immediately assayed for ADP-ribosyl transferase activity.

[2] Toxin A was obtained from our laboratory and is electrophoretically pure. Toxin corresponding to hydroxyapatite gradient peak I was applied to a G-100 sephadex column, yielding enzymatic activity in the void volume (G-100 void peak). Toxin corresponding to HA gradient peak III, when applied to G-100 sephadex yielded the G-100 50K dalton peak.

[3] NRS, normal rabbit serum; anti A, rabbit toxin A antiserum; anti S, rabbit S antisera.

a wheat germ extract protein with a molecular weight of 90-100K daltons. The 30K dalton enzyme is very selective, and only ADP-ribosylates to a minor extent an additional protein at about 50K daltons (not itself). The 60K enzyme, however, also ADP-ribosylates many other proteins, all smaller than the major substrate at 90-100K daltons. When these reaction products are compared with those observed with partially purified toxin (post DE-52 batch elution or HA batch elution) or with crude toxin (culture supernatant) we see that all preparations label the 90-100K material, but that some variation is seen in the labeling profile of the smaller proteins. Variation in labeling profiles of substrates observed on autoradiograms of reaction products are observed when SDS is present in the wheat germ extract in very low concentrations, or when the pH of the reaction is varied. At pH 6.0, the pH optimum for total enzymatic activity of crude toxin S, both 60K and 30K dalton toxins obtained from analytical SDS gel slices primarily label one species at 90-100K daltons, although many other proteins are labeled by the 60K toxin. At pH 7.5 (the pH or our wheat germ extract) labeling is specific only with the 30K toxin; material consisting primarily of 60K toxin demonstrated much less specificity, that is, the 90-100K proteins becomes no longer the major substrate. Post DE-52 and Post HA batch eluted toxins demonstrate very similar reaction product profiles. Interestingly, another strain of pseudomonas, WR 487, that produces toxin S also demonstrated ADP ribosylation of a major band at 90-100K daltons. Also, strains 388 infected burned mouse skin extracts demonstrate measureable S toxin activity whose reaction products are nearly identical to those observed with crude toxin from culture supernatant. A summary of S characteristics is presented in Table II.

During the course of these studies we have experienced losses in S enzymatic activity either due to inactivation or actual loss of S protein. Furthermore our results suggest that in culture supernatants. S aggregates or associates with lipids so that it is stable but does not enter many chromatographic resins. We have therefore investigated the possibility of using detergents to solubilize and stabilize crude S in order to develop more efficient methods of purification. Solubilization was monitored by chromatography of S on a Bio Gel A-0.5m column which has an exclusion limit of 500K daltons. Untreated crude S elutes as a peak at the void volume in a pattern identical to Dextran blue 2000. Furthermore, incubation (37°C for 30 min.) of S with 0.5% SDS or Triton X-100 with or without reducing agents failed to alter this elution profile even when the test detergent was included in the chromatographic buffer. However, when the detergent Brig-58 was incorporated in the chromatographic buffer (final concentration 0.1%) toxin S eluted as a broad peak between the void volume and hemaglobin. Thus Brig-58 appears to solubilize toxin S. Furthermore Brig-58 also seems to stabilize the enzymatic activity of S. Of the three detergents tested Brig-58 is the most hydrophobic therefore we intend to test other detergents such as Triton X-165, which are still more hydrophobic than Brig-58. These experiments may lead to an improved method of purifying toxin S.

In summary, 1) We have developed a procedure which greatly purifies S, but with a significant loss in enzyme acitivity.

2) We have demonstrated that a 30K fragment or subunit is also present, not neutralizable by anti S, which resembles fragment

Table 11. Characteristics of Partially purified Toxin S.

Characteristic	30K dalton enzyme[1]	60K dalton enzyme[1]	G-100 void peak[2]	G-100 50K peak[2]
Enzyme activity neutralized by anti S antiserum	No	No	Yes	No
Enzyme activity neutralized by anti A antiserum	No	No	No	No
Enzyme activity potentiated by Urea-DTT pretreatment	No	No	No	No
Labeling pattern of substrate by autoradiography	specific	promiscuous		
Molecular weight by SDS electrophoresis, daltons	28-30K	60K	60K	30K
Molecular weight by non denaturing gel filtration, daltons	48-50K	>>100K	>>100K	48-50K

[1] Material eluted from analytical SDS-polyacrylamide gel slices.

[2] Material eluted from G-100 columns run under non denaturing conditions.

A of exotoxin A. Moreover, it is not neutralized by anti A antibody.

3) The 30K fragment behaves as if it has a molecular weight of ~50K daltons on nondenaturing gel filtration, and demonstrates microheterogeneity on non denaturing polyacrylamide gel electrophoresis.

4) The 60K toxin material enzymatic activity resembles crude S activity in culture supernatant; ie. it is neutralizable by anti S, is excluded into the void volume in gel chromatography and has a multitude of substrates.

5) Toxin S from culture supernatants shows similar substrate specificity as toxin S produced in vivo.

D. Discussion

Purification of toxin by conventional ion exchange and chromatographic techniques appears to yield two forms of toxin, one with a multiplicity of substrates (and a molecular weight of 60K daltons) and another whose molecular weight is slightly less than 30K daltons with only one major substrate. Our preliminary evidence of multiple substrate labeling by Toxin S produced in vivo (S activity associated with burn mouse skin extracts) certainly implies that the 60K dalton form is found in vivo. The similarity in labeling patterns of wheat germ extracts by toxin S produced in vivo, in vitro, or after binding out of culture supernatant and elution from DEAE cellulose and hydroxyapatite indicates that the toxin behaves similarly when isolated from different sources and when partially purified. However, the variation in toxin S behavior toward substrate labeling under different reaction conditions (eg. variation in pH or the presence of a detergent) indicates that the 60K enzyme form is more sensitive to environmental factors during assay with crude wheat germ substrate preparations than the less promiscuous 30K dalton enzyme.

The yield of toxin in conventional purification schemes is based on calculated maximal incorporation of NAD^+ into the crude wheat germ extract for a given set of standard assay conditions. This is necessarily an extrapolation which may not be an applicable procedure since we are dealing, at least after removal from culture supernatant, with two enzyme entities demonstrating completely different kinetics. Far more labeled NAD^+ may be incorporated using crude preparations of toxin (predominately 60K daltons) in our wheat germ extracts (since substrate is not limiting with the assay conditions used) than by 30K dalton toxin (where substrate is limiting). At this time it is not known if seperation of 30K from 60K material (by SDS gel electrophoresis, hydroxyapatite gradient chromatography, or non denaturing gel filtration or electrophoresis) materially reduces the enzyme activity of the 60K toxin. It is not yet known if "add-back" experiments will demonstrate a synergistic effect.

We are currently investigating the possibilities that the 30K enzyme is a fragment generated during purification, or that it is simply selectively pulled out of cultures supernatant (ie. present at very low levels).

Neutralization experiments demonstrate that the nondetergent treated 30K material has enzymatic activity that is not neutralized by antisera made against partially purified S (post DEAE cellulose, then DEAE sephadex gradient) or toxin A. We know that unlike post hydroxyapatite toxin (stage II or III, fig. 1), the toxin used to raise antisera will not penetrate non denaturing gels and is excluded by gel filtration columns, and behaves like a large aggregate. However, SDS gel electrophoresis of this crude post DEAE cellulose toxin used in preparation of antisera demonstrates both 30K and 60K toxin peaks (activity may be eluted from SDS fels). This implies that it is possible that the 30K toxin may very well be, like fragment A of exotoxin A, a fragment of the toxin molecule. This fragment may be either normally buried in the intact molecule or simply not particularly immunogenic. The possibility should not be overlooked that pseudomonas proteases may have nicked the native form of the toxin during purification or growth in culture, and that the ion exchange process has allowed selective purification of an enzymatically active fragment of the toxin. We are pursuing the production of specific 30K and 60K antibodies in rabbits for the purpose of elucidating the relationship between these species. Furthermore, our preliminary results on solubilization and stabilization of toxin S with Brig-58 will be extended.

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Appendix #1

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Production of Exoenzyme S During *Pseudomonas aeruginosa* Infections of Burned Mice

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Antisera which distinguished between *Pseudomonas aeruginosa* exoenzyme S and toxin A neutralized the adenosine diphosphate ribosyl transferase activity of the homologous, but not the heterologous, enzyme. Skin extracts and sera from burned mice infected with the exoenzyme S-producing strain *P. aeruginosa* 388 contained adenosine diphosphate ribosyl transferase activity that was not found in skin extracts or sera from uninfected mice. On the basis of immunological reactivity and enzymatic properties, the adenosine diphosphate ribosyl transferase activity present in skin extracts and sera from *P. aeruginosa* 388-infected mice was identified as exoenzyme S. Active elongation factor 2 levels in tissues from strain 388-infected mice were normal at 24 h postinfection, indicating that strain 388 does not produce detectable amounts of toxin A in vivo. An unexpected finding in this investigation was the presence of exoenzyme S-inactivating activity in the sera from some nonimmunized animals.

Pseudomonas aeruginosa is an opportunistic pathogen that produces a wide variety of extracellular products that may contribute to its pathogenicity (17, 18). Toxin A has the potential to be a major virulence factor (1, 3, 10, 11, 17, 19, 21-25). Toxin A exerts its lethal effect by inhibiting protein synthesis in the same manner as diphtheria toxin, i.e., by catalyzing the transfer of the adenosine diphosphate (ADP) ribose moiety of nicotinamide adenine dinucleotide onto eucaryotic elongation factor 2 (EF-2) (6, 9-11).

A second extracellular protein (exoenzyme S) produced by some strains of *P. aeruginosa* has recently been shown to have ADP-ribosyl transferase activity (13). Exoenzyme S differs from toxin A in that it does not ADP-ribosylate EF-2 but, rather, modifies one or more different proteins present in eucaryotic cell extracts (13). Furthermore, exoenzyme S is not precipitated or neutralized by A antitoxin (13). The enzymatic activity of S is partially destroyed by pretreatment with urea and dithiothreitol (DTT) (13), whereas such pretreatment potentiates the enzymatic activity of toxin A (16, 28).

No studies have been done to determine if exoenzyme S plays a role in *P. aeruginosa* infections. As a first step in evaluating this possibility, the present study was undertaken to determine if exoenzyme S is produced in vivo. A second objective was to further examine the immunological relationship between exoenzyme S and toxin A.

MATERIALS AND METHODS

Bacterial strains. *P. aeruginosa* strain 388 was kindly provided by B. Minshew, University of Washington School of Medicine, Seattle, Wash., and strain PA-103 was provided by P. V. Liu, University of Louisville School of Medicine, Louisville, Ky. Strain 388 has been shown to produce exoenzyme S, but not toxin A, in vitro (13). Strain PA-103 produces toxin A, but not exoenzyme S, in vitro. The strains were serotyped as described by Fisher et al. (7). Relevant characteristics of these strains are shown in Table 1.

Reagents. Nicotinamide adenine dinucleotide ([^{14}C]adenine) was purchased from Amersham Corp. DTT, histamine, casein, elastin-congo red, and nitrilotriacetic acid were purchased from Sigma Chemical Co. Norit A neutral-activated charcoal was obtained from Fisher Scientific Co.

Growth and exoenzyme S production by *P. aeruginosa* 388. The medium used for the growth of strain 388 was as previously described (13). An overnight culture (4 ml) inoculated into 100 ml of medium in each 2-liter flask was grown at 32°C with vigorous shaking. At 22 h, the cells were removed by centrifugation at 10,000 × *g* for 20 min at 4°C, and the supernatants were pooled.

Toxin purification. Strain 388 supernatant was diluted with 3 volumes of ice-cold water, 50 g of equilibrated diethylaminoethyl (DE-52)-cellulose (Whatman, Inc., Clifton, N.J.) was added, and the mixture was then stirred for 1 h. The DE-52 was

removed by filtration onto Whatman no. 1 filter paper and washed with 2 liters of 50 mM NaCl–10 mM tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 8.0, and then exoenzyme S was eluted with 300 mM NaCl–10 mM Tris-hydrochloride, pH 8.0. The eluate was filter sterilized (Nalge Co., Rochester, N.Y.) and concentrated by ultrafiltration, using a PM-10 membrane (Amicon Corp., Lexington, Mass.), and the buffer was reequilibrated to 50 mM NaCl–10 mM Tris-hydrochloride, pH 8.0. Approximately 15 ml (1.1 mg/ml) of this material was applied to a diethylaminoethyl-Sephadex A-25 column (2.5 by 8.0 cm). A linear gradient from 50 to 400 mM NaCl was applied in 10 mM Tris-hydrochloride, pH 8.0. The major active peak at 200 mM NaCl was pooled and concentrated. These procedures resulted in a 30-fold purification of exoenzyme S which contained 0.48 mg of protein per ml with a ratio of optical density at 280 nm to that at 260 nm 1.4. Aliquots were frozen at −70°C.

P. aeruginosa PA-103 was used as a source of toxin A, which was produced and purified as previously described (28).

Preparation of specific antisera. A 1-ml mixture consisting of equal parts of Freund complete adjuvant (Difco) and 200 μg of partially purified exoenzyme S per ml in phosphate-buffered saline was injected into each adult male New Zealand rabbit as follows: 0.1 ml subcutaneously in each hind foot, 0.4 ml subcutaneously in the back, and 0.4 ml intramuscularly. The animals were then injected three times every 2 weeks, using the same sites and doses, in Freund incomplete adjuvant (Difco). Ten days after the last injection, the rabbits were bled, and the separated serum (rabbit S antiserum) was stored in small aliquots at −20°C. Purified toxin A was used to immunize rabbits and a sheep as previously described (12).

Purification of toxin A antibody. Toxin A was coupled to cyanogen bromide-activated Sepharose 4B (Pharmacia Fine Chemicals Inc., Piscataway, N.J.) as described by March et al. (19). The toxin A-Sepharose column buffer consisted of 50 mM Tris-hydrochloride, pH 8.0, 200 mM NaCl, and 1 mM ethylenediaminetetraacetate. Immunoglobulin was precipitated from sheep antitoxin A with ammonium sulfate (12) and then dialyzed against column buffer. Ten milliliters was applied to the toxin A-Sepharose column at 4°C. Material unbound after 20 min was washed out with column buffer, and then bound material was eluted with 50 mM glycine-hydrochloride, pH 3.2. The eluates (antitoxin A immunoglobulin) from three to four columns were pooled, concentrated to 1 mg/ml on an Amicon PM-30 membrane, and then reequilibrated with column buffer by ultrafiltration and stored at 4°C.

ADP-ribosyl transferase activity. Ten microliters of each sample was incubated at 25°C with 25 μl of wheat germ extract (150 μg) (12), 25 μl of reaction buffer (5 mM Tris-hydrochloride, pH 8.2, 0.1 mM ethylenediaminetetraacetate, 40 mM DTT), and 5 μl of nicotinamide adenine dinucleotide ([^{14}C]adenine) (280 mCi/mmol; 12.5 μCi/ml). Reaction mixtures containing mouse skin extract or serum were incubated for 30 min, whereas those with toxin A or exoenzyme S were incubated for 5 min. All reactions were stopped by the addition of 0.1 μl of 10% trichloroacetic acid and processed, and radioactivity was measured as previously described (12). Where indicated, samples were preincubated with an equal volume of 8 M urea and 2% DTT for 15 min at 25°C (12) and then assayed for ADP-ribosyl transferase activity.

Enzyme neutralization by sera. All sera were heat inactivated (56°C for 15 min) before their ability to neutralize ADP-ribosyl transferase activity was tested. Culture supernatants of *P. aeruginosa* strains 388 and PA-103 were used as a source of crude exoenzyme S and toxin A, respectively. Rate-limiting concentrations of these enzymes were obtained by diluting crude exoenzyme S 1:30 and crude toxin A 1:3 before use. Crude toxin A was then activated with urea and DTT (12). Skin extracts and serum samples from mice were used undiluted. Neutralization was examined by preincubating equal volumes of the appropriate serum and sample for 15 min at 37°C and then assaying ADP-ribosyl transferase activity as described above.

Experimental burn infection model. A burned mouse model, previously described (22, 26), was used. Female Swiss white mice (strain NIH-NMRI CV) weighing 20 ± 2 g were anesthetized with methoxyflurane (Penthrane; Abbott Laboratories, North Chicago, Ill.) and subjected to a 10-s alcohol flame burn involving 15% of the total body surface. Mice were injected subcutaneously in the burn area immediately after burn trauma with two 50% lethal doses of the appropriate strain, which resulted in fatal infections in about 90% of the mice 50 ± 10 h postinfection. Control animals consisted of anesthetized, nontraumatized, or burned mice injected subcutaneously with 0.5 ml of sterile phosphate-buffered saline. At appropriate intervals postinfection, mice were sacrificed by cervical dislocation and blood was obtained by cardiac puncture. Full-thickness specimens of burned skin (or unburned skin from appropriate control animals) were removed, and skin extracts were prepared as described by Saelinger et al. (24).

Extraction and quantitation of mouse organ EF-2. Livers, kidneys, and spleens were removed from mice immediately after they were sacrificed, and the tissues were frozen at −70°C. EF-2 was extracted from and quantitated in tissue homogenates by the method of Gill and Dinius (8) as modified by Iglewski et al. (11).

Other methods. Protein concentrations were determined by the method of Bradford (4), modified by using a commercial reagent (Bio-Rad Protein Assay Dye Reagent Concentrate) purchased from Bio-Rad Laboratories, Richmond, Calif. Bovine gamma globulin (Bio-Rad) was used as the standard. Proteolytic activity in crude supernatants of *P. aeruginosa* strains PA-103 and 388 was determined by the method of Kunitz (14) as modified by Wretlind and Wadstrom (29), using casein as the substrate. Elastase activity was quantified with elastin-congo red as a substrate as previously described (29).

RESULTS

Specific neutralization of exoenzyme S activity. To determine if exoenzyme S was produced in vivo, we used an immunological method to specifically identify this enzyme and distin-

guish it from toxin A. Antisera obtained from rabbits immunized with exoenzyme S neutralized the enzymatic activity of exoenzyme S but not that of toxin A. The enzymatic activity of exoenzyme S was not neutralized by rabbit A antitoxin, which completely neutralized the toxin A enzymatic activity. Since these antisera specifically neutralized the enzymatic activity of the homologous, but not the heterologous, enzyme, they could be utilized to identify the enzymatic activity in an unknown sample.

We also tested the neutralizing ability of A antitoxin which had been raised in sheep by immunization with pure toxin A. Surprisingly, this sheep antitoxin A neutralized the enzymatic activity of both toxin A and S exoenzyme. However, when examined, it was found that the preimmunization serum from this sheep neutralized S enzymatic activity, but not toxin A enzymatic activity. The anti-S titer of the pre- and post-toxin A immune sheep sera were identical. Anti-S activity copurified with gamma globulin during ammonium sulfate precipitation but did not copurify with specific antitoxin A immunoglobulin when it was purified on a toxin A-Sepharose 4B affinity column. Anti-S activity was also found in other (four of six) normal sheep sera, one of five normal rabbit sera, and two of six normal mouse sera (data not shown). It is interesting that none of the normal sera tested neutralized the enzymatic activity of toxin A.

In vivo production of exoenzyme S. The 50% lethal dose of strain 388 was markedly reduced when mice were burned (Table 1). The 50% lethal dose of strain 388 in normal (unburned) mice was 2.0×10^6 organisms, in contrast to a 50% lethal dose of 1.1×10^2 organisms in a burned mouse.

Skin extracts from burned mice that were infected with *P. aeruginosa* strain 388 contained ADP-ribosyl transferase activity that was not found in skin extracts from uninfected control mice (Fig. 1). The enzyme activity was present in the burned infected mouse skin extracts at the earliest time postinfection (18 h) that we tested and remained relatively constant from 18 to 48 h postinfection. Whereas there was a wide range of ADP-ribosyl transferase levels in the skin extracts of individual burned infected mice, 37 of 39 samples from infected mice had enzyme levels higher than those from all 49 control animals (Fig. 1).

The average level of ADP-ribosyl transferase activity in the sera from burned infected mice at 18 h postinfection was equal to that in the sera from control noninfected mice (Fig. 2). Levels of ADP-ribosyl transferase activity in the sera from infected mice increased markedly at 24 h postinfection and continued to increase linearly through 48 h. The average enzyme levels in sera from noninfected control mice did not change significantly over the 48-h period (Fig. 2). Enzyme levels similar to those of noninfected control mice were found in sera and skin extracts from mice infected with the toxin A- and exoenzyme S-negative strain WR-5 (data not shown).

Identification of the ADP-ribosyl transferase activity in samples from burned infected mice. The ADP-ribosyl transferase activity in skin extracts and sera from burned, strain 388-infected mice was further character-

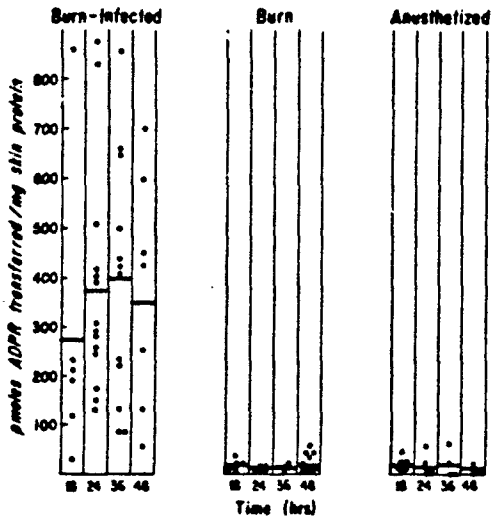

FIG. 1. *ADP-ribosyl transferase activity in skin extracts of burned mice infected with P. aeruginosa strain 388 and in skin extracts of control noninfected mice that were anesthetized and burned or anesthetized only. The horizontal lines represent the mean ADP-ribosyl transferase activity of skin extracts for each group of mice.*

TABLE 1. *Characterization of P. aeruginosa strains 388 and PA-103*

Strain	Source	Serotype	Toxin A	Exoenzyme S	Protease	Elastase	LD_{50} (CFU)*	
							Normal mice	Burned mice
388	Burn wound	1	−	+	+	+	2.0×10^6	1.1×10^2
PA-103	Sputum	2	+	−	+	−	1.8×10^6	1.2×10^3

* LD_{50}, 50% lethal dose; CFU, colony-forming units.

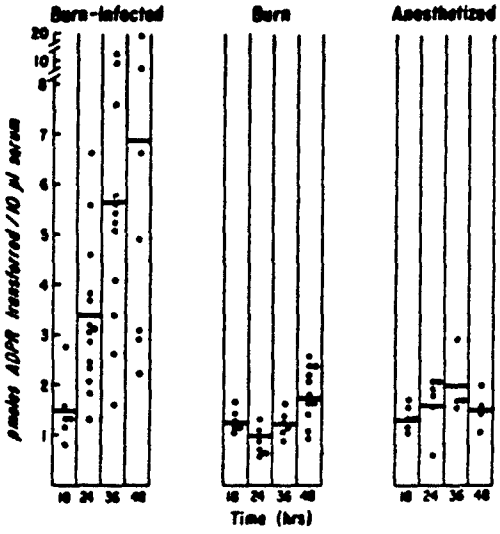

FIG. 2. *ADP-ribosyl transferase activity in sera from burned mice infected with P. aeruginosa strain 388 and in sera from control noninfected mice that were anesthetized and burned or anesthetized only. The horizontal lines represent the mean ADP-ribosyl transferase activity of sera for each group of mice.*

ized. Most of this enzyme activity was neutralized by exoenzyme S antiserum but not by A antitoxin (Table 2). The ADP-ribosyl transferase activity in sera from control (noninfected) mice was not neutralized by either A antitoxin or S antiserum (Table 2). These data indicate that most of the ADP-ribosyl transferase present in skin extracts and sera of strain 388-infected mice was due to exoenzyme S.

The treatment of skin extracts or sera from burned infected mice with urea and DTT partially destroyed the enzymatic activity (Table 3). To determine if the skin extracts or sera contained a factor which might alter these enzymes, crude exoenzyme S or crude toxin A was preincubated in skin extracts or sera from burned noninfected mice at 25°C for 15 min before assaying their enzymatic activities. The preincubation of these enzymes in uninfected mouse sera or skin extracts did not alter their enzymatic properties (Table 3, controls).

Active EF-2 levels in organs from *P. aeruginosa* 388-infected, burned mice. In agreement with previous reports (21, 22, 25), the levels of active EF-2 in tissues from burned mice infected with strain PA-103 were markedly decreased at 24 h postinfection (Table 4). In contrast, active EF-2 levels in burned mice infected with strain 388 were normal in the livers, kidneys, and spleens at 24 h postinfection (Table 4). At 48 h postinfection, only small decreases in the active EF-2 levels in the livers were found in strain 388-infected moribund animals (data not shown).

DISCUSSION

While investigating possible immunological cross-reactivity between exoenzyme S and toxin A, we found that sera from some nonimmunized animals inactivated S enzyme activity. The sera from one of five rabbits, five of seven sheep, and two of six mice partially neutralized S enzymatic activity (data not shown). Furthermore, a sheep immunized with purified toxin A had equal titers of anti-S activity in the pre-bleed and in the immune (antitoxin A) serum. The exoenzyme S-inactivating factor in this antitoxin A serum

TABLE 2. *Neutralization of ADP-ribosyl transferase activity in skin extracts and sera from mice*

Skin extracts/sera	Neutralization[a]	
	A antitoxin	S antiserum
Skin extracts: 388-infected burned mice		
Skin no.		
3	−	+ (87)
22	−	+ (84)
23	−	+ (82)
39	−	+ (86)
56	−	+ (87)
Sera: 388-infected burned mice		
Serum no.		
21	−	+ (53)
37	−	+ (80)
38	−	+ (78)
40	−	+ (58)
41	−	+ (73)
Sera: control (burned noninfected mice)		
Serum no.		
45	−	−
47	−	−
Sera: control (anesthesized only) mice		
Serum no.		
31	−	−
50	−	−

[a] Numbers in parentheses represent percentage of skin extract or serum ADP-ribosyl transferase activity that was neutralized by A antitoxin or S antiserum as compared to the ADP-ribosyl transferase activity of a sample treated with an equal volume of 0.9% saline containing 0.1 mg of bovine serum albumin per ml for 15 min at 37°C. Samples showing <10% reduction over controls were considered negative (−), and those with >10% reduction were scored positive (+).

TABLE 3. *Effect of urea and DTT on the ADP-ribosyl transferase activity of mouse skin extracts and sera*

Skin extracts/sera	ADP ribose incorporated (pmol)[a]	
	+ Water	+ Urea, DTT
Control skin extracts or sera + toxin A or exoenzyme S[b]		
Skin extract + toxin A	0.8	12.4
Skin extract + exoenzyme S	8.5	4.9
Serum + toxin A	1.7	13.4
Serum + exoenzyme S	11.9	5.5
Skin extracts from burned infected mice		
Skin no.		
3	28.0	15.4
23	48.1	32.1
37	60.0	45.9
39	50.9	29.0
56	25.9	19.6
Sera of burned infected mice		
Serum no.		
19	14.3	8.6
21	13.3	8.2
23	9.5	6.5
37	26.7	17.0
41	12.1	4.5

[a] Per 10 μl of skin extract or serum.

[b] Crude toxin A (10 μl) or crude exoenzyme S (10 μl of a 1:10 dilution) were preincubated with 90 μl of skin extract or sera for 1 h at 37°C before being tested for ADP-ribosyl transferase activity.

TABLE 4. *Comparison of the active EF-2 levels in tissue extracts from burned mice infected with P. aeruginosa PA-103 or 388*[a]

Organ[c]	% Control active EF-2 levels[b]	
	PA-103 infection	388 infection
Liver	35	102
Kidney	83	95
Spleen	82	101

[a] Mice were sacrificed 24 h after being burned and infected.

[b] Control values were obtained using the appropriate tissue from anethestized and burned uninfected mice.

[c] Organs from six similarly treated mice were pooled.

copurified with gamma globulin during ammonium sulfate precipitation but did not copurify with specific antitoxin A immunoglobulin. Whether this anti-S activity is due to antibody remains to be determined.

By immunizing only rabbits whose preimmune sera contained no detectable anti-S or anti-A activity, we were able to develop a suitable exoenzyme S antiserum. In a previous report (13), the enzymatic activity of exoenzyme S was not neutralized by A antitoxin. This observation is confirmed in this report, and it is also demonstrated that the enzymatic activity of toxin A is not neutralized by exoenzyme S antibody. Thus, these specific antisera (anti-S or anti-A) can be used to identify the enzymatic activity in an unknown sample providing the preimmune sera are first examined to ascertain that they do not have anti-S activity.

Most extracellular bacterial products known to be virulence factors have been shown to be produced in vivo. We attempted to detect the in vivo production of exoenzyme S by *P. aeruginosa* strain 388, a strain that produces the enzyme in vitro (13). Exoenzyme S was produced in vivo in burned mice infected with *P. aeruginosa* 388 (Fig. 1 and 2). ADP-ribosyl transferase activity was detected in extracts of skin obtained 18, 24, 36, and 48 h postinfection (Fig. 1). This enzymatic activity was also detected in sera from burned mice infected with strain 388 at 24 h postinfection, and the mean levels increased approximately linearly through 48 h (Fig. 2). That the ADP-ribosyl transferase activity detected in the skin extracts and sera of burned infected mice was indeed due to exoenzyme S was shown by its specific neutralization by S antiserum but not by A antitoxin (Table 2). In addition, this enzymatic activity present in skin extracts and sera from strain 388-infected animals was decreased by pretreatment with urea and DTT (Table 3), which is characteristic of exoenzyme S but not of toxin A (13). Finally, in contrast to the reduction of active EF-2 levels in tissues from burned mice infected with toxin A-producing strains of *P. aeruginosa* (21, 25), levels of EF-2 in the livers, kidneys, and spleens of burned mice 24 h after infection with strain 388 were not altered in comparison to the levels of EF-2 in noninfected control mice (Table 4). These data (Tables 2–4) indicate that strain 388 does not produce detectable amounts of toxin A in vivo. Small decreases in active EF-2 levels were observed in tissues from animals infected 48 h previously with strain 388. These slight decreases seen with strain 388 were similar to decreases previously reported using the toxin A- and exoenzyme S-negative strain, WR-5, and presumably reflect nonspecific tissue degeneration in moribund animals (21).

Exoenzyme S levels in skin extracts and sera of the burned infected mice varied over a wide range (Fig. 1 and 2). When the sera of six normal mice were tested for exoenzyme S-neutralizing activity, two of six were capable of partially neutralizing the ADP-ribosyl transferase activ-

ity of exoenzyme S (data not shown). One explanation for the wide range of responses of individual animals could be the presence of preexisting antibodies.

In conclusion, we have shown that exoenzyme S is produced in vivo in animals infected with *P. aeruginosa* strain 388. It was further demonstrated that strain 388 was virulent for burned mice and that this was not due to production of detectable levels of toxin A. Thus, exoenzyme S may be a virulence factor of *P. aeruginosa*. However, information concerning its toxicity, its production by clinical isolates, and the protective capabilities of specific S antibodies in *P. aeruginosa* infections is required to evaluate the relative importance of exoenzyme S.

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Appendix #2

Factors that Influence the Production of *Pseudomonas aeruginosa* Exoenzyme S

Running Title: Production of Exoenzyme S

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ABSTRACT

A medium has been developed and culture conditions identified which result in high yields of exoenzyme S in liquid cultures of Pseudomonas aeruginosa strain 388. The optimum medium composition and culture conditions for S were found to be similar but not identical to those previously reported for toxin A production. The major difference between S and A production was that S production required the presence of a chelating agent, either nitrilotriacetic acid (NTA) or ethylenediamenetetraacetate (EDTA). The optimum concentration of NTA or EDTA was found to be 10 mM. Furthermore, bovine serum albumin (BSA), either partially purified or crystalline, markedly inhibited S yields whereas crystalline BSA has previously been shown to enhance toxin A yields. The addition of various amino acids resulted in increased S yields but monosodium glutamate (MSG) (100 mM) gave the highest S yields. Yields of S were increased by the addition of glycerol to 1%. As the iron concentration of the growth medium was increased to 2 μg/ml and above, S yields were markedly (80%) decreased. A low iron medium composed of a dialysate of trypticase soy broth supplemented with 10 mM NTA, 1% glycerol and 100 mM MSG gave the highest yields of S. Relatively high S yields were also obtained when strain 388 was cultured in some other basal medium similarly supplemented. The optimum culture conditions for high S yields were adequate aeration and a temperature of 32° C. Under these culture conditions exoenzyme S was first detectable at 10 h and maximum yields were found at 20-24 h.

INTRODUCTION

Exoenzyme S is a recently discovered adenosine diphosphate ribosyl (ADP-ribosyl) transferase that is produced by some strains of Pseudomonas aeruginosa (11). This enzyme catalyzes the transfer of the adenosine diphosphate ribose (ADPR) moiety of nicotinamide adenine dinucleotide (NAD^+) onto proteins present in extracts of eukaryotic cells (11). However, exoenzyme S does not ADP-ribosylate elongation factor 2 (EF-2) as does P. aeruginosa toxin A (8,9,11). The ADP-ribosyl transferase activity of exoenzyme S is not neutralized, nor the enzyme precipitated, by toxin A antibody (11). In addition, the enzymatic activity of exoenzyme S differs from toxin A in that S activity is destroyed rather than potentiated (as is toxin A's enzymatic activity) by pretreatment with urea and dithiothreitol (11).

Toxin A is not formed constitutively by A toxinogenic strains of P. aeruginosa. Aeration, a temperature of 32° C, glycerol and monosodium glutamate (MSG) are required to obtain maximum yields of toxin A (15). Nucleic acids and iron inhibit the production of toxin A but enhance the growth of P. aeruginosa (2,15). While the factors that regulate yields of toxin A have been studied, little is known about the regulation of exoenzyme S yields. The aim of this study was to identify factors which regulate the yields of exoenzyme S in cultures of P. aeruginosa and optimize the culture conditions and medium constituents in order to obtain sufficient material for purification and toxicity studies.

MATERIALS AND METHODS

Microorganisms. P. aeruginosa strain 388 was used throughout this study. Strain 388, the kind gift of B. Minshew, University of Washington, Seattle, Wa., was stored frozen in 10% skim milk at -70° C.

Reagents. NAD([^{14}C] adenine) at 280 mCi/mmol was purchased from Amersham/Searle Corp. Nitrilotriacetic acid (NTA), bovine serum albumin ([BSA]; both fraction V and crystalline), casein, monosodium glutamate (MSG), elastin-congo red and tricine (N-Tris[hydroxymethyl] methylglycine) were purchased from Sigma Chemical Co., St. Louis, Mo. Glycerol and citrate were purchased from Fisher Scientific Co., Fair Lawn, N.J. and ethylenediaminetetraacetate (EDTA) from Matheson, Coleman and Bell, Los Angeles, Ca.

Media and culture conditions. Trypticase soy broth (TSB) medium was prepared by dissolving 30 g trypticase soy broth (BBL, Cockeysville, Md.) in 1 l of distilled water. Dialyzed TSB (TSBD) was prepared as described by Liu (15). For one set of experiments, TSBD medium was deferrated and the residual iron concentration of the medium determined (20). Syncase medium was prepared as described by Finkelstein et al. (7) with the exception that no iron was added. A protease peptone (PP) medium was prepared as described by Pope (21). To prepare dialysed proteose peptone (PPD) medium, 20 g of protease peptone powder (Difco Lab, Detroit, Mich.) dissolved in 60 ml H_2O was dialysed against 1 l H_2O. The other components of Pope's medium, $MgSO_4$, 0.2 mg; Na_2HPO_4, 1.0 g; K_2HPO_4, 1.0 g; glucose, 2.0 g; and sodium lactate (12 ml of a 50% solution) were

then added to each liter of dialysate. PGT (pantothenate, glutamic acid and tryptophan) medium was prepared as described by Barksdale and Pappenheimer (1). Unless otherwise indicated, media were supplemented with 10 mM NTA, adjusted to pH 7.0, autoclaved, then sterile solutions of MSG and glycerol were added to a final concentration of 100 mM and 1%, respectively.

Bacterial growth was determined by removing a portion of the culture and measuring the turbidity at 540 nm (OD_{540}) in a Beckman spectrophotometer 20. Generally, the culture conditions were as follows: 10 ml of the appropriate medium in a 125 ml Erlenmeyer flask was inoculated with a 15 h culture of strain 388 to give 0.05 OD_{540} units. Incubation was for 20 h at 32°C in a reciprocating shaking water bath (Lab-line Instruments, Melrose, Ind.).

ADP-ribosyl transferase assay. Cytoplasmic soluble extracts were prepared from wheat germ as described by Chung and Collier (6). The ADP-ribosyl transferase assay (11) was performed at 25° C for 5 min in a mixture containing 25 µl of wheat germ extract, 25 µl of reaction buffer (5 mM Tris(hydroxymethyl)aminomethane-hydrochloride (Tris. HCl), pH 8.0), 5 µl of ([^{14}C] adenine) NAD at 280 mCi/mmol (12.5 µCi/ml) and 5 µl of a 1:10 dilution of crude culture supernatant in 0.9% saline. The reaction was stopped by the addition of 0.1 ml of 10% trichloroacetic acid. The precipitates were collected, washed and radioactivity was measured (10).

Protease assay. Protease activity in culture supernatants was determined by a previously described (22) modification of the method of Kunitz (13) using casein as the substrate. One unit of proteolytic activity was defined as a change of one optical density (280 nm) unit using the assay method of Wretlind and Wadstrom (22).

RESULTS

Effect of NTA on exoenzyme S yields and protease activity. Callahan has previously shown that the presence of NTA in the culture medium inhibits the enzymatic activity of P. aeruginosa proteases (5). For this reason, NTA was added to TSBD medium in an attempt to detect toxin A in cultures of Elek negative (3) strains of P. aeruginosa. Some of the strains, in the presence of NTA, produced an ADP-ribosyl transferase (exoenzyme S) that was distinct from toxin A (11).

The effect of varying the NTA concentration of the medium (TSBD) on bacterial growth and exoenzyme S yields was investigated (Fig. 1). NTA (0-20mM) did not effect bacterial growth. At NTA concentrations greater than 40 mM there was a slight decrease in the final yields of bacteria. In contrast to its minimal effect on growth, NTA had a dramatic effect on S yields. When NTA was omitted from the culture medium, ADP-ribosyl transferase activity was barely detectable (Fig. 1). Addition of from 1 mM to 10 mM NTA to TSBD medium increased S yields, while NTA concentrations greater than 20 mM decreased S yields. However, S activity was still detectable at concentrations of NTA as high as 100 mM (Fig. 1). While there was a broad peak of NTA concentrations (5-20 mM) at which high yields of S were produced, the optimum concentration of NTA was 10 mM (Fig. 1).

In light of Callahan's previous report (5), it seemed possible that NTA increased S yields by inhibiting the enzymatic activities of P. aeruginosa proteases, which might otherwise degrade S. To test this

possibility, the effect of varying concentrations of NTA in the medium on S yields and protease activity was examined. As the concentration of NTA in the medium was increased, the protease activity decreased while S yields increased (Fig. 2). Addition of NTA to 4 mM resulted in a complete loss of detectable protease activity. However, S yields continued to increased as the concentration of NTA increased from 4 through 10 mM (Fig. 1 and 2).

Ammonium sulfate [$(NH_4)_2SO_4$] has also been used to inhibit the activity of proteases in cultures of P. aeruginosa (16). When ammonium sulfate (1-400 mM) was added to TSBD medium in place of NTA, only barely detectable yields of exoenzyme S were found (data not shown).

Since NTA is a potent chelating agent (5,17) the effect of other chelating agents on S yields were examined (Table 1). The chelating agents tested were tricine, citrate and EDTA. The addition of (0.1 mM-20 mM) tricine or citrate to the medium in place of NTA resulted in negligible S yields. The addition of EDTA to the medium resulted in S yields equal to the yields obtained when 10 mM NTA was present (Table 1 and Fig. 1). However, EDTA inhibited bacterial growth and pigment production by strain 388. The concentration of NTA or EDTA which gave maximum S yields was 10 mM and in subsequent experiments the media were supplemented with 10 mM NTA.

Effect of amino acids on S yields. A number of amino acids were added to TSBD to determine their effect on S yields. Among those tested were alanine, arginine, aspartic acid, glycine, isoleucine, and glutamic acid. Of these amino acids, aspartic acid and glutamic acid resulted in maximal S yields when added to TSBD medium, while the addition of arginine gave 50% maximal yields and alanine, glycine or isoleucine

resulted in S yields that were 10-20% maximal (data not shown). These amino acids are expensive and some of them are difficult to dissolve. A substitute, MSG, was found that was both inexpensive and water soluble at high concentrations. Increasing the concentration of MSG in the medium from 0 mM to 50 mM resulted in a substantial increase in bacterial growth (Fig. 3). At concentrations above 50 mM, MSG had no obvious effect on growth. The addition of MSG to TSBD medium increased S yields. As the concentration of MSG in the medium increased from 10 to 100 mM, S yields increased proportionally (Fig. 3). At concentrations greater than 100 mM, MSG had an inhibitory effect on S yields although even at 200 mM the yields were still about 40% maximal (Fig. 3).

To determine if protein could substitute for MSG, the effect of various concentrations of BSA (fraction V or crystalline) on S yields and bacterial growth was determined. The addition of either partially pure (fraction V) or crystalline BSA in place of MSG resulted in decreased yields of exoenzyme S, while at the same time stimulating bacterial growth (Fig. 4). The data showing the inhibitory effects of fraction V and crystalline BSA were very similar (Fig. 4). In light of these results (Fig. 3 and 4), MSG at its optimum concentration of 100 mM was added to media used in subsequent experiments.

Effect of glycerol and S yields. Because glycerol had been found to enhance yields of toxin A (15), in previous experiments we added glycerol (1% final concentration) to the growth medium. To determine if glycerol stimulated S yields and if so to determine its optimum concentration, the effect of various concentrations of glycerol on S yields and bacterial growth was examined. The addition of glycerol

to a final concentration of 0.25% markedly increased bacterial growth but had no effect on S yields (Table 2). Glycerol at a final concentration of 0.5% doubled the S yields and at 1.0%, S yields were maximal. In the presence of 2.0% glycerol the S yields decreased (Table 2). Glycerol (1%) was added to media for all subsequent experiments.

Effect of iron on S yields. Since the yields of a number of bacterial products, including P. aeruginosa pigments and toxin A, are decreased by increasing the iron concentration of the medium (2,4,12), the effect of iron on S yields in cultures of strain 388 was examined. Increasing the concentration of iron in the medium resulted in an increase in bacterial growth (Fig. 5). In contrast, as the iron concentration of the medium increased from 0.05 µg/ml to 1.0 µg/ml the yields of exoenzyme S decreased. No further decreases in S yields occurred when the concentration of iron was increased above 2.0 µg/ml. Exoenzyme S yields in medium containing 2 or 5 µg iron/ml were decreased by about 80% as compared to medium containing 0.05 µg/ml (Fig. 5).

Culture conditions and S yields. Bacterial growth and S yields were both increased in shaking cultures of strain 388 as compared to stationary cultures (Table 3). Bacterial growth was increased 2-3 fold in the shaking cultures while exoenzyme S yields were increased about 50 fold as compared to yields in stationary cultures. The optimum temperature of incubation for the production of S in stationary cultures was 32-35° C. In shaking cultures the optimum was 32° C and 80% maximal S yields were found at 30° C. Yields of S in shaking cultures incubated at 25°-37° C were higher than S yields in stationary cultures even when the latter were incubated at their optimum temperatures (32-35° C) (Table 3).

Rate of exoenzyme S release and bacterial growth. Strain 388 was cultured in TSBD medium supplemented with 10 mM NTA, 100 mM MSG and 1% glycerol. At 2 h intervals, samples were removed from the culture and examined for growth and exoenzyme S yields (Fig. 6). The generation time of strain 388 during exponential growth was 25 min as calculated by the method of Miller (18). Strain 388 reached stationary phase at 18 h. Exoenzyme S was first detected at 10 h and the rate of release remained approximately constant through 20 h. Maximal yields of S were found late in the bacterial growth cycle at 20-24 h (Fig. 6).

Other media and S yields. Yields of exoenzyme S in other media were compared to those in TSBD medium (Table 4). Some of these media, such as syncase, PGT and PP have been used for the production of other bacterial toxins (1,7,21). Of the media tested, S yields were maximal in TSBD. However, relatively high S yields were obtained when strain 388 was cultured in some of the other media. The dialysate of TSBD resulted in much higher yields of S than TSB medium. Conversely, PP medium (non-dialysed) resulted in higher S yields than PPD (Table 4). When NTA was omitted from any of the media, S activity was barely detectable (data not shown). Exoenzyme S yields were not simply a function of bacterial growth since there was no consistent relationship between bacterial yields and S yields in the media tested (Table 4).

DISCUSSION

A medium has been developed which results in reproducible high yields of exoenzyme S in cultures of P. aeruginosa strain 388. This medium (Table 5) is relatively inexpensive, easily prepared and has the advantage that it contains no high molecular weight components that could otherwise complicate the purification of exoenzyme S. Although the medium was optimized for S yields using strain 388, other exoenzyme S producing strains of P. aeruginosa produce high S yields in this medium (data not shown). Routinely, a flask to volume ratio of at least 10 to 1 was used to obtain adequate aeration. Furthermore, yields of exoenzyme S were approximately 50 times higher in shaking cultures than in stationary cultures (Table 3). Exoenzyme S yields consistent with the yields described in this paper have also been obtained in fermentor scale cultures of strain 388 using the fermentation conditions described by Leppla for toxin A production (14). The optimum incubation temperature was 32° C, although significant amounts of S were obtained in shaken cultures incubated between 30-37° C (Table 3).

The medium and conditions that have been developed for optimizing yields of exoenzyme S (Table 5) are similar to those developed by Liu for toxin A (15). Among the similarities are the use of TSBD as the base medium, the requirement for MSG and glycerol, and culture conditions such as the temperature of incubation and aeration. Furthermore, maximum yields of S are obtained in growth medium containing relatively low concentrations of iron and yields decrease in the

presence of high iron concentrations (Fig. 5). A similar relationship has previously been shown for iron and toxin A yields (2). In addition to having similar requirements for growth medium composition and culture conditions, the initial appearance and rate of release of exoenzyme S (Fig. 5) are similar to that previously shown for toxin A (2).

Differences were observed in the regulation of exoenzyme S and toxin A yields. The addition of NTA to the growth medium was found to be required for S production (Fig. 1 and 2) but not toxin A production (5,15; and M. Bjorn, unpublished observation). NTA was required for S production regardless of the kind of growth medium used. NTA is a metal chelating agent which has been used by Callahan (5) to inhibit the activity of Pseudomonas proteases, which may be metalloenzymes (19). NTA at a concentration as low as 4 mM completely inhibited the protease activity of P. aeruginosa strain 388 (Fig. 2). Thus it would be tempting to conclude that NTA maximizes S yields by inhibiting the activity of proteases which in the absence of NTA destroy S. A comparison of the concentration of NTA required for maximum S yields (10 mM) and that (4 mM) required to inhibit detectable protease activity (Fig. 1 and 2) suggest that inhibition of protease activity is not the only mechanism by which NTA enhances S yields. This suggestion is supported by our observation that another protease inhibitor, $(NH_4)_2SO_4$ (16), could not substitute for NTA in the growth medium. At a variety of concentrations of $(NH_4)_2SO_4$, in the absence of NTA, strain 388 produced no detectable exoenzyme S. Further evidence for the suggestion that inhibition of protease activity is not the only mechanism by which NTA enhances S yields was obtained by the use of an extracellular protease deficient mutant of strain 388. Despite its extra-

cellular protease deficient phenotype, this mutant still requires 10 mM NTA in the medium in order for yields of exoenzyme S to be maximal (data not shown). Since NTA is a potent chelating agent it may be making one or more ions, which inhibit S yields, unavailable to the bacteria. Of those chelating agents examined only EDTA was able to substitute for NTA in the growth medium (Table 1). The concentration of EDTA or NTA which gave maximal S yields was 10 mM (Fig. 1 and Table 1). Although not identical, the stability constants of NTA and EDTA for a variety of metals are generally similar, whereas those of the other chelating agents tested (Table 1) generally differ from NTA and EDTA (17). The effect of NTA and EDTA is not only to reduce the iron concentration of the medium. In extensively deferrated medium, where the iron concentration was growth limiting, NTA or EDTA was still required for high S yields (data not shown). This result suggests that another metallic ion in addition to iron may regulate exoenzyme S yields. The exact mechanism by which NTA or EDTA enhance S yields is not yet known.

In addition to the requirement for NTA or EDTA in the growth medium, production of exoenzyme S differs from toxin A production in its response to the presence of crystalline BSA. Yields of S decreased in the presence of crystalline BSA (Fig. 6) whereas yields of toxin A have been shown to increase in the presence of crystalline BSA (15). The mechanism whereby BSA inhibits S yields is not yet known. Whether other proteases will have a similar effect on S yields remains to be determined.

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Table 1. Effect of metal chelators on bacterial growth and exoenzyme S yields in cultures of P. aeruginosa 388[a]

Conc. of chelator	Tricine		Citrate		EDTA	
	Bacterial growth	ADPR incorporated	Bacterial growth	ADPR incorporated	Bacterial growth	ADPR incorporated
mM	(OD_{540})	(pmoles)	(OD_{540})	(pmoles)	(OD_{540})	(pmoles)
0.1	8.4	1.0	9.0	0.6	8.4	0.5
1.0	8.7	0.6	9.0	0.4	8.4	0.6
10.0	8.4	0.7	8.7	0.6	6.0	667
20.0	8.4	0.7	9.0	0.4	5.7	620

[a]Media were supplemented with 100 mM MSG and 1% glycerol. Cultures were incubated for 22 h with shaking at 32° C.

Table 2. Effect of glycerol on bacterial growth and exoenzyme S yields in cultures of *P. aeruginosa* 388[a]

Conc. of glycerol (%)	Bacterial growth (OD_{540})	ADPR incorporated (pmoles)
0	5.7	353
0.25	9.4	363
0.50	11.1	665
1.0	12.0	781
2.0	11.7	543

[a]Media were supplemented with 10 mM NTA and 100 MSG. Culture conditions were as stated in Table 1.

Table 3. The effect of temperature and aeration on bacterial growth and exoenzyme S yields in cultures of *P. aeruginosa* 388[a]

	Shaking cultures		Stationary cultures	
Temperature (C)	Bacterial growth (OD_{540})	ADPR incorporated (pmoles)	Bacterial growth (OD_{540})	ADPR incorporated (pmoles)
25	6.6	60	1.2	1
30	11.7	399	4.8	7
32	11.7	505	3.6	10
35	7.8	182	3.0	11
37	7.8	148	3.3	5

[a]Media were supplemented with 10 mM NTA, 100 mM MSG and 1% glycerol. The cultures were incubated for 22 h.

Table 4. Bacterial growth and exoenzyme S yields in various media[a]

Medium	Bacterial growth (OD_{540})	ADPR incorporated (pmoles)
TSB	15.6	15
TSBD	9.6	691
PP	10.8	526
PPD	6.9	371
Syncase	8.1	274
PGT	7.2	127

[a]Each medium was supplemented with 10 mM NTA, 100 mM MSG and 1% glycerol. Culture conditions were as stated in Table 1.

Table 5. Medium composition for the production of *P. aeruginosa* exoenzyme S[a]

Trypticase soy broth dialysate	30 g/liter H_2O
Nitrilotriacetic acid	10 mM
pH to	7.0
Monosodium glutamate	100 mM
Glycerol	1%

[a]The optimum culture conditions are: flask size to volume ratio of at least 10:1, adequate aeration and an incubation temperature of 32° C for 22 h.

FIGURE LEGENDS

Fig. 1. Effect of various concentrations of NTA on the yields of exoenzyme S in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (△) bacterial growth as measured at 540 nm. Media were supplemented 100 mM MSG and 1% glycerol. Cultures were incubated for 22 h with shaking at 32° C.

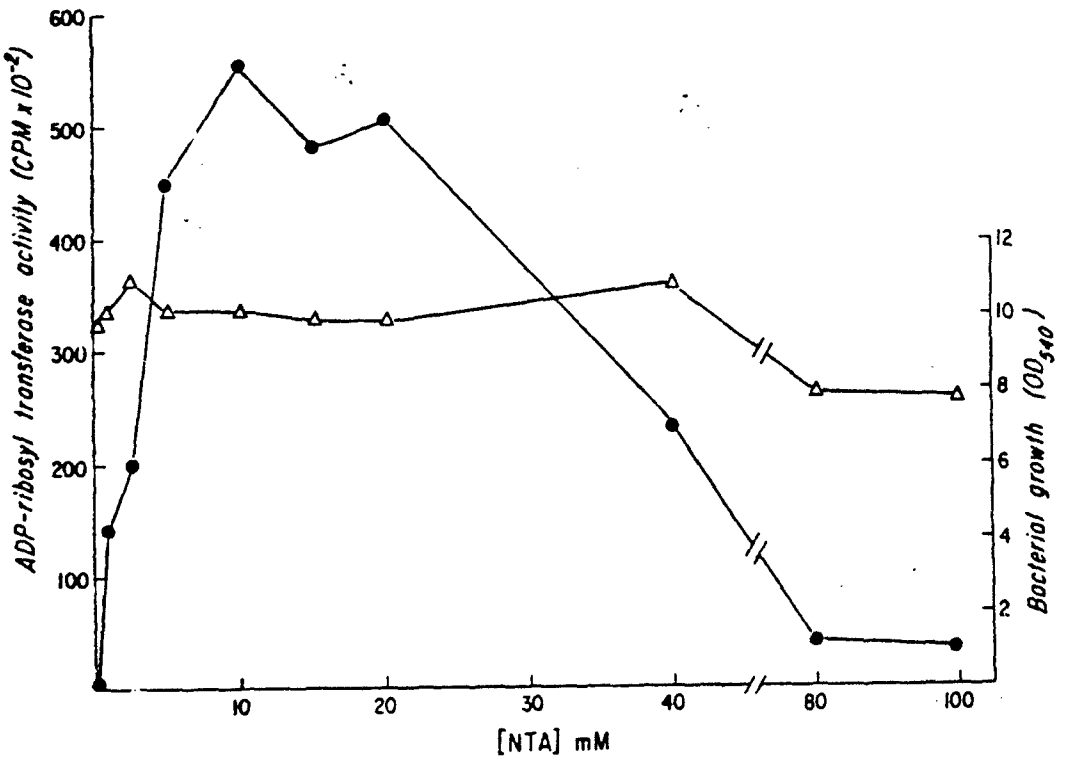
Fig. 2. Effect of increasing concentrations of NTA on the yields of exoenzyme S and protease activity in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (□) protease activity in proteolytic units/ml. Media supplementation and cultural conditions were as stated in Fig. 1.

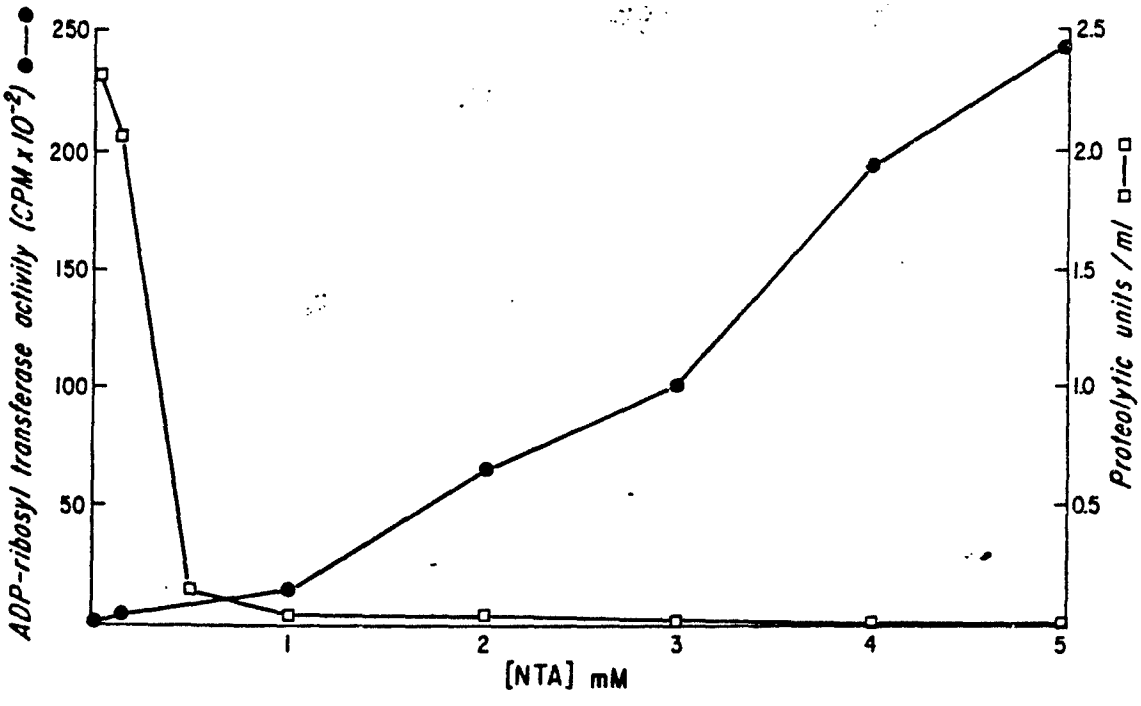
Fig. 3. Effect of various concentrations of MSG on the yields of exoenzyme S in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (△) bacterial growth as measured at 540 nm. Media were supplemented with 10 mM NTA and 1% glycerol. Cultural conditions were as stated in Fig. 1.

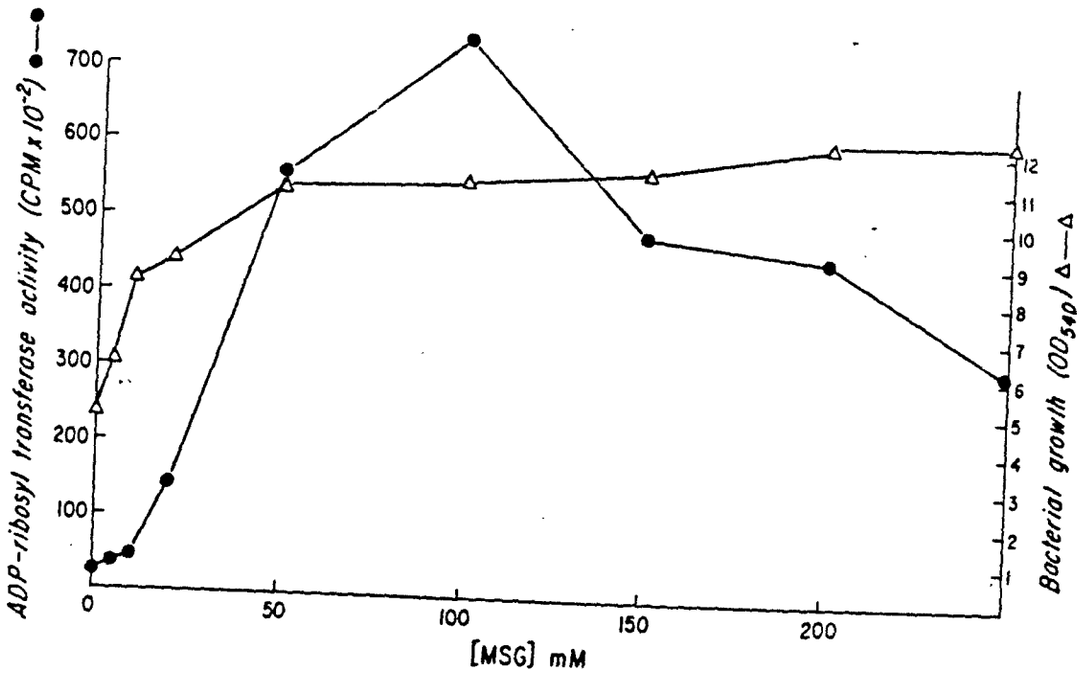
Fig. 4. Effect of increasing concentrations of BSA on the yields of exoenzyme S in cultures of P. aeruginosa 388. S yields in the presence of (A) impure (fraction V) BSA and (B) crystalline BSA. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (△) bacterial growth as measured at 540 nm. Media supplementation was as stated in Fig. 3 and cultured conditions as stated in Fig. 1.

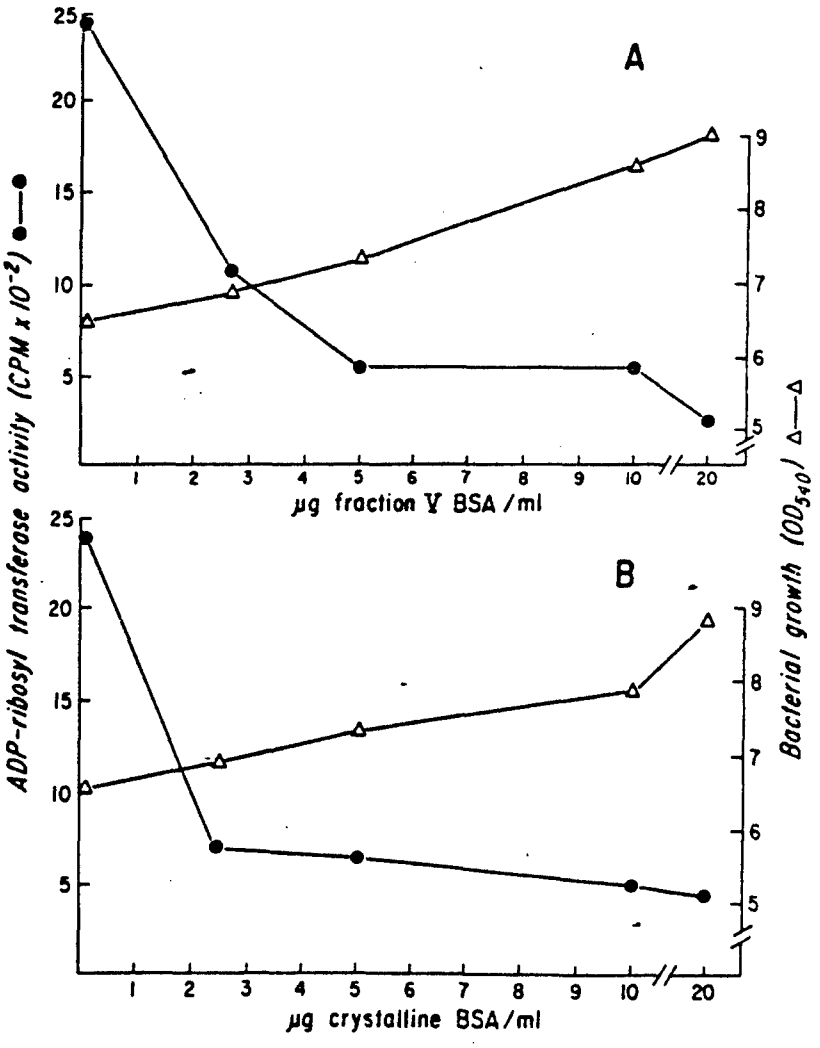
Fig. 5. Effect of various concentrations of iron on the yields of excenzyme S in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl transferase activity (cpm x 10^{-2}); (△) bacterial growth as measured at 540 nm. Media were supplemented with 10 mM NTA, 100 mM MSG and 1% glycerol. Cultural conditions were as stated in Fig. 1.

Fig. 6. Rate of release of exoenzyme S in cultures of P. aeruginosa 388. Symbols: (●) ADP-ribosyl trarsferase activity (cpm x 10^{-2}); (△) bacterial growth as measured at 540 nm. Media were supplemented as stated in Fig. 5.


ADP-ribosyl transferase activity (CPM x 10⁻²)
Bacterial growth (OD540)
[NTA] mM
600
500
400
300
200
100
12
10
8
6
4
2
10
20
30
40
80
100


ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Proteolytic units / ml □—□
250
200
150
100
50
2.5
2.0
1.5
1.0
0.5
1
2
3
4
5
[NTA] mM


ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Bacterial growth (OD540) Δ—Δ
700
600
500
400
300
200
100
0
50
100
150
200
[MSG] mM
12
11
10
9
8
7
6
5
4
3
2
1


A
B
ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Bacterial growth (OD540) △—△
μg fraction V BSA /ml
μg crystalline BSA/ml
25
20
15
10
5
9
8
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6
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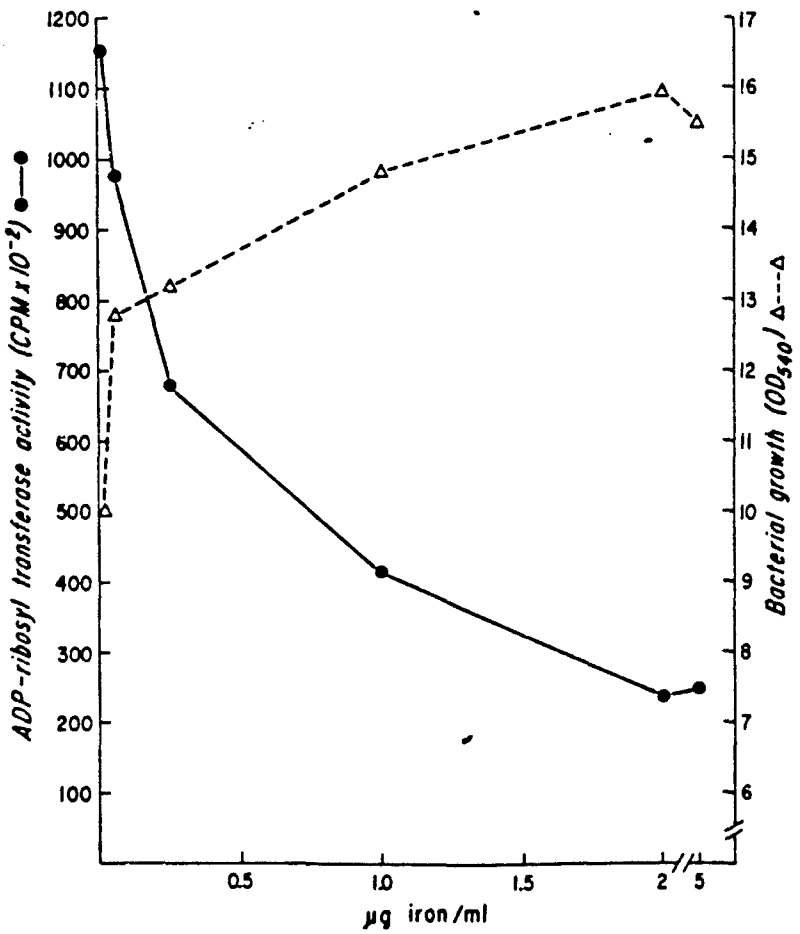
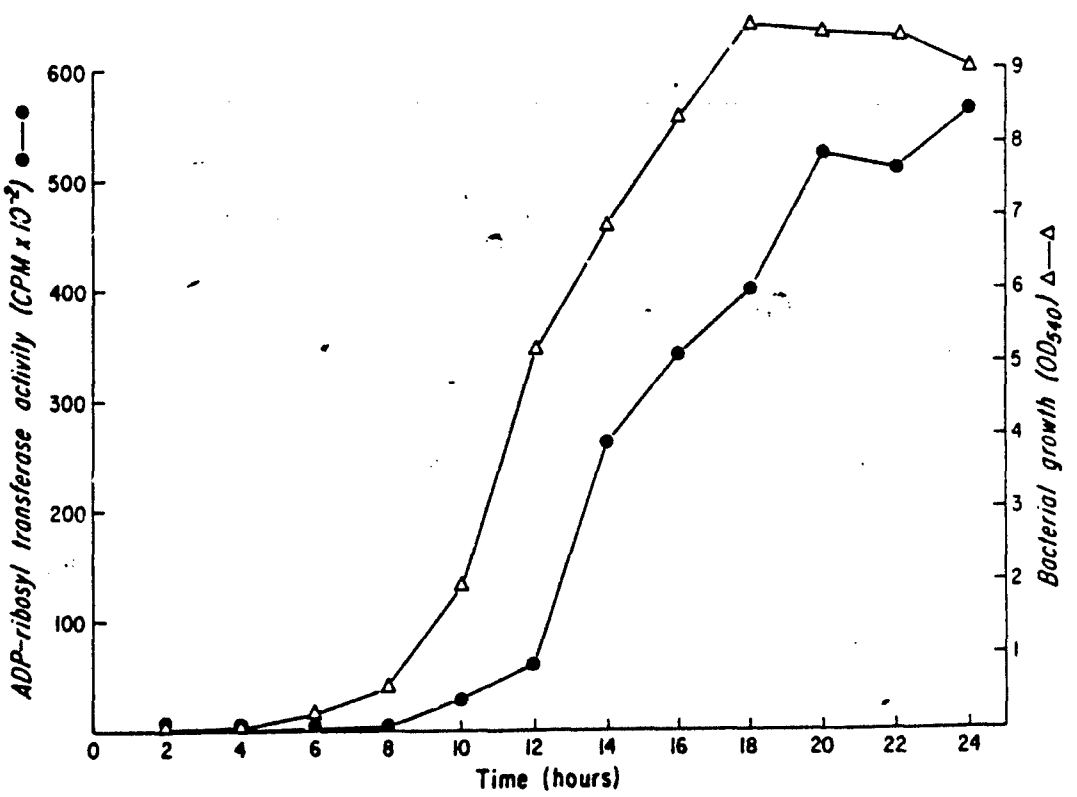
2
3
4
5
6
7
8
9
10
20


ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Bacterial growth (OD540) Δ---Δ
1200
1100
1000
900
800
700
600
500
400
300
200
100
17
16
15
14
13
12
11
10
9
8
7
6
0.5
1.0
1.5
2
5
µg iron/ml


ADP-ribosyl transferase activity (CPM x 10⁻²) ●—●
Bacterial growth (OD540) △—△
Time (hours)
600
500
400
300
200
100
0
2
4
6
8
10
12
14
16
18
20
22
24
9
8
7
6
5
4
3
2
1

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Physiological Sciences

Appendix #3

Effects of pseudomonas toxin A, diphtheria toxin, and cholera toxin on electrical characteristics of turtle bladder

(sodium, bicarbonate, and chloride transport/transepithelial electrical potential, conductance, and short-circuiting current)

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ABSTRACT Rapidly developing changes in the short-circuiting current (I_{sc}), conductance (G), and potential (PD) of turtle bladders in Na-rich or Na-free media are seen after the mucosal addition, at 10 nM, of each of three toxins that contain ADP-ribosylation activity: *Pseudomonas aeruginosa* toxin A, diphtheria toxin, and cholera toxin. Toxin A irreversibly decreased the I_{sc}, PD, and G of bladders in Na-rich media and the I_{sc} and PD of bladders in Na-free media. Diphtheria or cholera toxin reversibly increased I_{sc} and PD (not G), but only in Na-free media. The effects of toxin A in the turtle bladder, like those in other host cell systems, were eliminated by preexposure of this toxin to heat, specific antitoxin, or dithiothreitol and urea. Because exposure to this last condition increases the ADP-ribosylation activity of toxin A, it is suggested that the proenzyme is the required transport-inhibiting form of toxin A. The effects of all three toxins occurred rapidly, possibly before any of the possible intracellular ADP-ribosylation reactions are initiated. Whereas a recognition binding of toxin to receptors on the apical membrane completely accounts for the reversible effects of diphtheria or cholera toxin, this and additional toxin–membrane interactions (e.g., translocation) are needed to account for the irreversible effects of toxin A.

The lethality of *Pseudomonas aeruginosa* toxin A in experimental animals (1–4) and tissue cultures (5, 6) has been attributed to an inhibition of protein synthesis resulting from the ADP-ribosylation of elongation factor 2 (7, 8). This mechanism is similar to that demonstrated for diphtheria toxin (9, 10). The pathogenetic sequence leading to cell destruction by toxin A is thought to be initiated by an extracellularly located, proenzymatic form of the whole toxin molecule and terminated by an intracellularly located, enzymatically activated form of the toxin or its *a* fragment (8, 11, 12). The mechanism by which any molecule of this size can penetrate a plasma membrane and gain access to the cytoplasm is not yet fully established. One recently proposed mechanism, "receptor-mediated pinocytosis" (13), is consistent with data on toxin A, diphtheria toxin (13–16), and other macromolecules (13).

Whatever its exact nature, the penetration of a membrane by these toxins should be accompanied by concomitant changes in certain measurable characteristics of that membrane—e.g., in the transmembrane permeability or electrical conductance (G or 1/R), the potential difference (PD), the flux of penetrant ions, or the short-circuiting current (I_{sc}). Such characteristics are not readily or directly measurable in the host cell systems that have been studied in relation to these toxins. These electrical values however can conveniently be measured in several ion-transporting epithelia such as the turtle bladder (17–19). This tissue was therefore chosen as a potential host cell system on which the membrane effects induced by toxin A or diphtheria toxin were studied.

In what follows, we show that the mucosal addition of toxin A induces rapid irreversible changes in I_{sc}, PD, and G in bladders bathed by Na-rich or Na-free media; that the proenzyme form rather than the enzymatically active form of toxin A is required for eliciting these changes; and the qualitatively different but nonetheless distinct and reproducible effects are evoked by the mucosal addition of diphtheria or cholera toxin to bladders in Na-free media.

METHODS

Ion Transport. Excised bladders of *Pseudemys scripta* turtles were mounted between two bathing fluids in a Rehm–Ussing chamber and continuously short-circuited. The I_{sc}, instantaneous open-circuit PD, and G were monitored as described (17).

For studies on Na transport, the mucosal surface of the bladder was bathed in a Na-rich, Cl-free, HCO_3-free solution (Na_2SO_4 Ringer's solution) and the serosal surface, by a Na-rich, Cl- and HCO_3-containing solution (Na Ringer's solution). Under such conditions, the I_{sc} has been shown to approximate the net rate of Na reabsorption (18). The composition of the serosal fluid was the following (mM): NaCl, 25; $NaHCO_3$, 20; Na_2SO_4, 27.5; $MgSO_4$, 0.8; K_2SO_4, 2; K_2HPO_4, 0.61; KH_2PO_4, 0.14; $CaSO_4$, 2.0; glucose, 11; sucrose, sufficient to make the final osmolality 220 mosM/kg. The composition of the mucosal fluid was the same except that SO_4 was substituted for Cl and HCO_3.

For studies on anion transport, bladders were bathed on both surfaces by identical Na-free solutions containing Cl and HCO_3 and having the same composition as the serosal Na Ringer's solution except that choline was substituted for Na (choline Ringer's solution). Under these conditions, the I_{sc} has been shown to approximate the sum of the net fluxes of Cl and HCO_3 from mucosa to serosa (17, 19). Ouabain (0.1 mM) was added to the serosal fluid of all bladders bathed in Na-free Ringer's solution.

Pseudomonas and Diphtheria Toxins, Antitoxin, and Pseudomonas Endotoxin. Toxin A was isolated and purified from culture supernatants of *P. aeruginosa*, strain PA-103 (20) as described (21). Antitoxin was prepared in rabbits as described (22). Diphtheria toxin (Connaught, Toronto, ON, lot D-279) was further purified as described (11). The purity of these exotoxins was established by their migration as a single band in sodium dodecyl sulfate/polyacrylamide gels (11). The amount of toxin A existing as the proenzyme was estimated by deter-

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Abbreviations: G, conductance; PD, potential difference; I_{sc}, short-circuiting current.

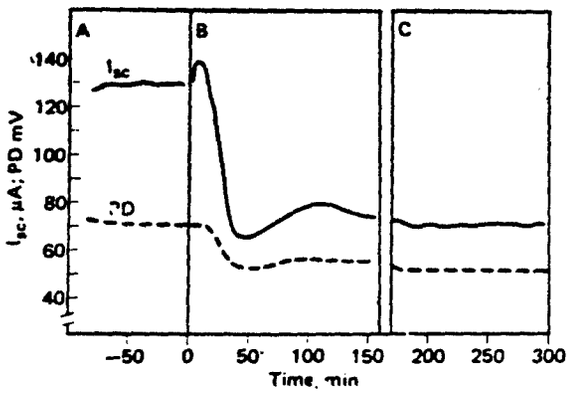

FIG. 1. Effect and irreversibility of effect of toxin A on I_{sc} and PD of a turtle bladder in Na-rich media. Mucosal fluid, Na_2SO_4 Ringer's solution (Cl-free, HCO_3-free). Serosal fluid, Na Ringer's solution (with Cl and HCO_3). Toxin concentration was decreased by 10 consecutive half-replacements (5 ml) of mucosal fluid with equal volumes of toxin-free mucosal fluid at 160–170 min. (*A*) Control period. (*B*) Toxin A at 46 nM. (*C*) Toxin-free (<10 pM).

mining the ADP-ribosylation activity of the toxin before and after treatment with 4 M urea and 1% dithiothreitol (11, 21). Endotoxin was purified from *P. aeruginosa* strain PA-103 by the phenol/water method of Westphal *et al.* (23). Cholera toxin was obtained from Schwarz/Mann.

RESULTS

The addition of toxin A to the mucosal fluid (final concentration, 10 nM) of bladders in Na-rich media was followed by a short period (3–4 min) in which no electrical changes occurred and then by a longer period (10–15 min) in which I_{sc} and G increased by 10–15% and PD remained unchanged. In the next 30 min, PD as well as I_{sc} and G decreased to ≈60% of the pre-toxin (control) levels, and these decreased levels were maintained for the final 5 hr in this and in 14 similar experiments (Fig. 1). The mean values of I_{sc}, PD, and G during these post-toxin periods (1–5 hr) were significantly less than those during the control periods (Table 1).

The percentage decrease in I_{sc} was related to the final mucosal concentration of toxin A in eight experiments. Minimal decreases (15%) were found at toxin concentrations of 0.5 nM (two bladders) and maximal decreases (40–50%), at toxin concentrations of 5 nM. Further increases in concentration produced no further effects.

The toxin A-induced changes were found to be irreversible in six of the bladders in Na-rich media. Once the decreased levels of I_{sc}, PD, and G were reached, they could not be restored or changed by dilution of the mucosal concentration of toxin A (10 nM) to subthreshhold concentrations of 10–0.01 pM (Fig. 1C).

Whereas addition of toxin A to the serosal fluid failed to change any of the electrical characteristics, its subsequent addition to the mucosal fluid was followed by the usual changes in I_{sc}, PD, and G (Fig. 2) in five experiments. This preferred mucosal sidedness suggests the presence of toxin A-reactive sites on the apical (mucosal) membrane.

Table 1. Effect of toxin A on I_{sc}, PD, and 1/G (electrical resistance)

	I_{sc}, μA	PD, mV	1/G, kohms
Before	93.0 ± 11.5	72.1 ± 5.3	0.91 ± 0.11
After	50.4 ± 6.2	51.8 ± 1.7	1.20 ± 0.20
MPD*	−44.6 ± 2.9	−29.5 ± 2.5	+22.7 ± 2.6

Data are shown as means (±SEM) before and 2–4 hr after mucosal addition of toxin (10 nM) to 15 bladders in the Na-rich bathing system.

* Mean (±SEM) of the individual percentage changes. All values of MPD were significantly different from 0 (i.e., $P < 0.001$).

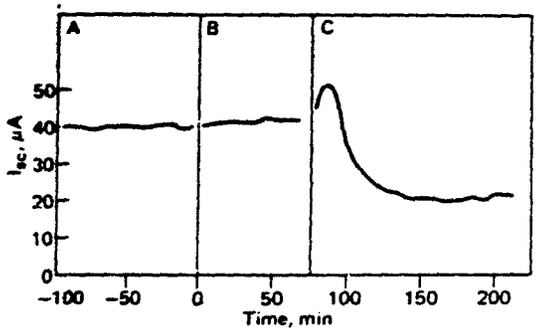

FIG. 2. Preferential mucosal-sidedness of action of toxin A in a bladder bathed by Na-rich medium (described in legend of Fig. 1). (*A*) Control. (*B*) Toxin A, 40 nM in serosal fluid. (*C*) Toxin A, 10 nM in mucosal fluid.

Whereas the mucosal addition of a preheated (100°C) aliquot of toxin A failed to produce any change, the subsequent mucosal addition of a nonheated aliquot of the same toxin was followed by the expected decreases in I_{sc} and PD (Fig. 3). These findings are in harmony with previously reported effects of heating toxin A—namely, inactivation of its toxic effects in whole animals and in cells in tissue culture (1, 6).

In the next experiments, a 2-fold molar excess of specific antibody against toxin A (from the serum of immunized rabbits) was mixed with toxin A and allowed to incubate at 37°C for 15 min. The addition of this mixture (cooled to 25°C) to the mucosal fluid bathing one of a mated pair of half-bladders in Na-rich media evoked no changes in any of the electrical values in the following 1 hr, at which time the mucosal fluid was removed and replaced with a fresh mucosal fluid free of toxin or antitoxin. The addition of toxin A to this fresh mucosal fluid (final concentration, 10 nM) was followed by decreases in the levels of I_{sc} (Fig. 4), G, and PD (not shown). The addition of another aliquot of the same toxin A preparation to the mucosal fluid of the mated half-bladder (i.e., the one not previously exposed to antitoxin alone or to the toxin/antitoxin mixture) also produced decreases in I_{sc}, PD, and G after 30 min (see Figs. 1–3 for comparison). The premixture of toxin A with specific antitoxin provided complete protection of the bladder from the effects of this toxin in one experiment and partial protection in a second. Premixture with normal rabbit serum provided no protection from the effects of toxin A; and mucosal additions of toxin A antibody alone were without effect on the electrical values of six bladders.

Because toxin A exists in a proenzymatic state as well as in an enzymatically activated state (8, 11), it was of particular interest to find that the lower the intrinsic ADP-ribosylation activity, the greater the toxin A-induced inhibition of I_{sc} and G in turtle bladders bathed by Na-rich media (Table 2). It was inferred that the proenzymatic form of toxin A (rather than the enzymatically activated form) interacts with the apical membrane. This inference was confirmed by dividing a single toxin A preparation (which had inhibited transport in the bladder) into separate aliquots, of which some were exposed to dithiothreitol and urea and others were not. The ADP-ribosylation activity of the treated toxin was 7.5-fold greater than that of the untreated toxin (Exp. 4, Table 2). The addition of the enzymatically activated aliquot of this toxin to the mucosal fluid produced only small and spontaneously reversible changes in the transepithelial electrical values of the bladder. However,

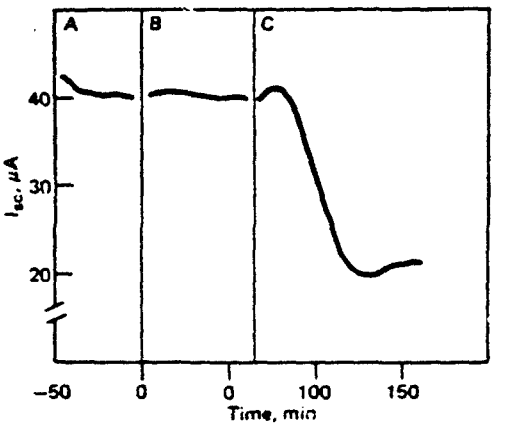

FIG. 3. Lack of effect of boiled toxin A on the I_{sc} of a turtle bladder in the Na-rich bathing system (described in legend of Fig. 1). (*A*) Control. (*B*) Boiled toxin A, 30 nM in mucosal fluid. (*C*) *B* plus native toxin A, 30 nM in mucosal fluid.

the subsequent addition of the untreated aliquot (the proenzymatic form) of the same toxin A preparation was followed by clear-cut, sustained decreases (42%) in I_{sc}, PD, and G across the bladder.

On the basis of the preceding data, it can be inferred that toxin A first accelerates and then irreversibly retards the flow of Na across the apical membrane (from the mucosa to the cell fluid). However, a specific preference of this toxin for Na path sites was ruled out when it was found that the mucosal addition of toxin A nullified the I_{sc} and PD without changing G across four bladders in Na-free media (Fig. 5). The serosal addition of toxin A was without effect in these bladders. Under these Na-free conditions, the magnitude of the I_{sc} has been shown to approximate the sum of the net Cl and HCO_3 reabsorption (17, 19); and the toxin A-induced electrical changes correspond precisely to those that would occur after a decrease in the net driving force(s) of the pumps for Cl and HCO_3 reabsorption (or proton secretion) in an electrical model of the bladder under Na-free conditions.

Because it appears that the mucosal surface of the bladder contains toxin A-reactive sites near the anion-selective paths (Fig. 5) as well as near the cation-selective paths (Figs. 1–3) of the apical membrane, it was of interest to determine whether such sites could react with other polypeptide toxins with ADP-ribosylation activity such as diphtheria (9, 10) or cholera toxin (24, 25) or even with lipopolysaccharidic toxins which lack

Table 2. Relationship between enzymatic activation of toxin A (four separate preparations) and the effect of each toxin preparation on I_{sc} across intact bladders in Na-rich medium

Toxin	Preincubation*	ADP-ribosylation activity, cpm/tube	Effect on Isc, decrease†
1	No	7,946	10
	Yes	9,294	ND
2	No	8,766	0
	Yes	7,786	ND
3	No	10,463	0
	Yes	9,552	ND
4	No	2,192	42
	Yes	16,323	0

* With dithiothreitol and urea.

† ND, transport effect of preincubated aliquot of toxin A was not tested; the transport effect of the untreated toxin A aliquot was tested in all four preparations.

this enzyme activity (e.g., the endotoxin of *P. aeruginosa*). In fact, each of these toxins did induce an electrical response in the turtle bladder; but the characteristics of each response differed qualitatively and quantitatively from those of the response to toxin A.

The mucosal addition of diphtheria toxin (final concentration, 10 nM) to bladders in Na-free media was followed within 1 min by a doubling of the PD and I_{sc} with little or no change in the transepithelial G (Fig. 6) in six experiments. These increased levels returned promptly to control levels after replacement of the toxin-containing with toxin-free fluid. No change was found after the serosal addition of diphtheria toxin to bladders in the Na-free media.

The mucosal addition of diphtheria toxin to bladders in Na-rich media was followed by rapidly developing [$t_{1/2} \approx 3$ min] increases in I_{sc} and G but only in the first one of six consecutive experiments on bladders under these bathing conditions. Serosal additions were without effect. We cannot yet explain these results because each batch of diphtheria toxin that failed to induce electrical changes in the five bladders bathed by Na-rich medium did induce an increase in the I_{sc} and PD in concomitant experiments on bladders in Na-free media.

Preliminary tests with cholera toxin were made in six experiments on bladders in Na-free media. The mucosal addition of this toxin (10 nM) was followed within 1–2 min by rapidly developing, reversible increases (doubling) in the I_{sc} and PD. This pattern, which is that of increasing the net driving force of all operative ion pumps in an electrical model of the bladder

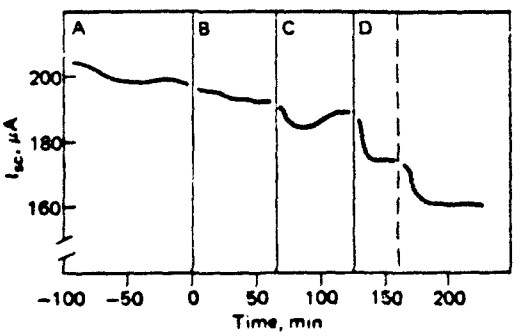

FIG. 4. Protective effect of specific antibodies against toxin A, and the subsequent effect of toxin A alone on the I_{sc} of a bladder in the Na-rich bathing system. Toxin was added at 100 min (5 nM) and again at 150 min to a final concentration of 10 nM. (*A*) Control. (*B*) Toxin plus antibody. (*C*) Washout period. (*D*) Toxin alone.

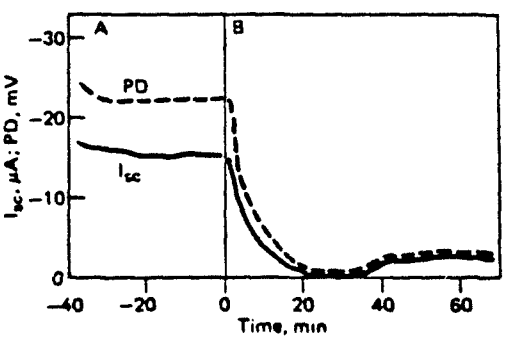

FIG. 5. Effect of toxin A on I_{sc} and PD of a turtle bladder bathed on both surfaces by identical Na-free (choline) Ringer's solution containing Cl and HCO_3. Negative values for PD and I_{sc} mean that the serosa was electronegative to the mucosa. (*A*) Control. (*B*) Toxin A, 10 nM in mucosal fluid.

bathed by Na-free fluids, is indistinguishable from that induced by diphtheria toxin (Fig. 6).

A pattern of rapidly developing increases in I_{sc} and PD, which occurs after the mucosal addition of norepinephrine (26, 27) or cyclic AMP derivatives (28) to bladders in Na-free media, is indistinguishable from that which occurs after cholera or diphtheria toxin (Fig. 6). These increases are thought to be mediated by the binding of toxin or norepinephrine to sites on an adenylate cyclase-activating element distinct from but adjacent to adenylate cyclase per se in the apical membrane of the turtle bladder. In this connection, Lefkowitz *et al.* (29) have invoked the presence of catecholamine binding sites on nonenzymatic, membrane-bound elements to account for the catecholamine-induced activation of adenylate cyclase in the membranes of avian erythrocytes. The inference that this toxin-binding, cyclase-activating element is a ganglioside is consistent with the recent data of Tosteson and Tosteson (30) who showed that cholera toxin increases the conductance of ganglioside (G_{M1})-impregnated lipid bilayers interposed between buffered NaCl solutions.

A purified preparation of the endotoxin of *P. aeruginosa* (23) was added to the Na-rich solutions (final endotoxin concentration, 5 μg/ml) bathing four pairs of bladders. The levels of I_{sc} and G increased slowly and after 30 min were 35% higher than control levels; these increased levels were maintained throughout the duration of the experiment (3 hr). No electrical changes were found after serosal addition of endotoxin.

The response of the bladder to endotoxin was initially slower than the response to toxin A, diphtheria toxin, or cholera toxin; and there was no secondary phase of decreased G such as that characteristic of the response to toxin A. It follows that neither the early nor the later phase of the toxin A-induced response (nor the responses to diphtheria toxin or cholera toxin) can be ascribed to the action of endotoxin contaminants.

DISCUSSION

The absolute requirement for and the first step in any effective interaction sequence between an extracellularly located bacterial toxin and a susceptible host cell is the reversible or irreversible binding of that toxin to recognition sites on the external surface of the plasma membrane of the host cell. This recognition binding alone changes the electrical as well as other properties of the target membrane from those characteristic of its control state to those characteristic of its toxin-occupied state. The steps following the initial recognition binding (which comprise membrane penetration, cellular entry, and intracellular reactions) depend on the nature of the specific toxin as well as that of the specific host cell type. Some of these steps also change the electrical properties of the target membrane, but only after the recognition binding of toxin has occurred.

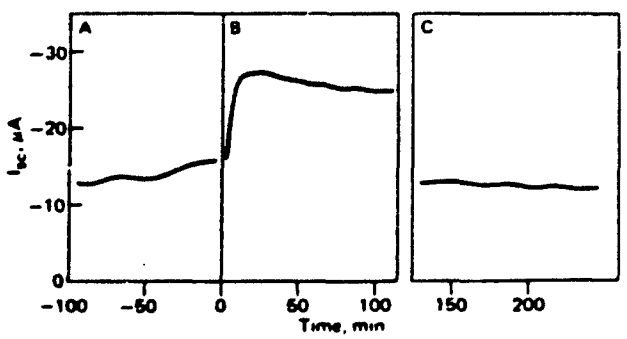

FIG. 6. Reversibility of the effect of diphtheria toxin on the I_{sc} of a bladder bathed by Na-free (choline) Ringer's solution containing Cl and HCO_3. Negativity of I_{sc} means that the serosa was electronegative to the mucosa. (*A*) Control. (*B*) Diphtheria toxin, 10 nM in mucosal fluid. (*C*) Toxin-free (<10 pM) mucosal fluid.

In the present study, a tentative hypothesis of toxin binding to recognition sites on the anion pump(s) or on the anion-selective conductances in series with these pumps in the apical membrane can account completely for the diphtheria toxin-induced (or cholera toxin-induced) increases in the PD and I_{sc} without requiring that either of these toxins enter the cytoplasm or even penetrate further into the matrix of the apical membrane. Evidence for the gangliosidic nature of the recognition sites for cholera toxin has been presented elsewhere (see *Results* and ref. 30).

On the other hand, the toxin A-induced changes in the electrical characteristics of bladders in Na-rich or Na-free media are only partially accounted for by invoking recognition binding as the sole interaction of this toxin with the apical membrane. Recognition binding alone can account for a monophasic change in conductance but cannot account for a biphasic change such as that found after the mucosal addition of toxin A to bladders in Na-rich media (Figs. 1–3). Nor can recognition binding alone account for an irreversible decrease in the PD and I_{sc} with an invariant G, as is found after a similar addition of toxin A to bladders in Na-free media (Fig. 5). In fact, all of the toxin A-induced decreases in G, PD, and I_{sc} are irreversible. Therefore, simple recognition binding is the first step but not the only step in the sequence of toxin A interaction(s) with the turtle bladder.

The occurrence of other steps such as membrane translocation or cellular entry in the interaction between toxin A and its target membrane is based on the following observed analogies. (*i*) Extracellularly placed toxin A does enter and destroy cells of whole animals and tissue culture lines (1, 5, 6), which suggests that this toxin can also migrate across the apical membrane to enter the turtle bladder cell. (*ii*) The ability of toxin A to induce electrical changes in the turtle bladder is eliminated by prior treatment of the toxin with heat, urea and dithiothreitol, or specific antibody, all of which have previously been shown to eliminate its toxic effects in whole animals or cultured cells (1, 5, 6, 11). (*iii*) When toxin A is activated so that its ADP-ribosylation activity is maximal, it produces little or no electrical change in the turtle bladder (Table 2). Thus, the proenzyme rather than the enzymatically activated form of toxin A is necessary for the proper initial binding to a specific membrane receptor and consequently for the subsequent events of transmembrane migration and possibly entry of toxin A into the turtle bladder cell as well as into other cell systems. (*iv*) The toxin A-induced changes in the electrical characteristics of the turtle bladder presumably reflect membrane changes that occur rapidly, possibly before any intracellular reaction can be initiated in this tissue. The latter claim is made with the reservation that the active elongation factor 2 content of toxin A-treated bladder cells remains to be determined. However, these analogies indicate the occurrence but not the exact mode of transmembrane migration or cellular entry of toxin A, which remains speculative at present.

In summary, three different ADP-ribosylation activity-containing toxins interact with and induce characteristic changes in the electrical characteristics of the turtle bladder. Additional experiments are needed to show how the distinctly different changes induced by toxin A or diphtheria toxin in the turtle bladder are related to the cellular destruction induced by these toxins in experimental animals and tissue cultures (1, 5, 6) and how the changes induced by cholera toxin in the turtle bladder are related to the cellular dysfunction induced by this toxin in man or experimental animals (31).

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BINDING OF DIPHTHERIA TOXIN TO PHOSPHOLIPIDS IN LIPOSOMES.

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Diphtheria toxin bound to the phosphate portion of some, but not all, phospholipids in liposomes. Of those tested, liposomes to which toxin did not bind consisted of dimyristoyl phosphatidylcholine (DMPC)/cholesterol (CHOL), and DMPC/CHOL plus 20 mol% (compared to DMPC) of phosphatidylinositol. In the order of relative effectiveness, inclusion of 20 mol% of the following lipids in the liposomes resulted in toxin binding: dipalmitoyl phosphatidic acid (calcium salt), dicetyl phosphate (DCP), phosphatidylinositol phosphate, cardiolipin, dipalmitoyl phosphatidic acid (sodium salt), and phosphatidylserine. Binding of toxin to liposomes consisting of phosphatidylcholine/CHOL/DCP was inversely related to the fatty acyl chain length of phosphatidylcholine (lauroyl > myristoyl > palmitoyl > stearoyl). The least binding occurred with liposomes in which phosphatidylcholine was replaced by sphingomyelin. Binding of toxin was inhibited by UTP, ATP, phosphocholine, p-nitrophenylphosphate, and phosphate-buffered saline, but not by uracil or normal saline. We conclude that diphtheria toxin binds specifically to the phosphate portion of certain phospholipids, and that binding may be influenced by the composition of adjacent phospholipids that do not bind toxin.

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